IMMUNITY TO POLIOMYELITIS IN MOTHERS AND THE NEWBORN AS SHOWN BY THE NEUTRALIZATION TEST*

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(Received for publication, June 10, 1930)

Evidence has been presented elsewhere relating to the age distribution of poliomyelitis which indicates that immunity to the disease is widespread, bearing a direct relationship to age and concentration of population and an inverse relationship to the incidence of the disease in all age groups.† It was inferred from this study that the virus of the disease has a similar widespread distribution (1). Neutralization tests on a series of individuals not giving a history of poliomyelitis were reported in a later communication (2). In an urban population im-

* This work was supported by the Harvard Infantile Paralysis Commission, a fund privately donated to the Vermont Department of Public Health, and a gift from the International Committee for the Study of Infantile Paralysis.

† While increasing immunity with increase in age is undoubtedly a major factor in determining the diminishing incidence of the disease with increase in age, there are indications that especially after adult age is reached a diminishing chance of adequate exposure is also a factor not only in the diminishing incidence of the disease but also in a diminution in the rate with which immunization occurs with increase in age. Diminishing chances of contact infection with age are also suggested by Chapin's figures for measles, which show a diminishing attack rate in susceptibles in families with cases with increase in age (5), as well as by Pope's studies of scarlet fever (6). The idea of diminishing chance of exposure with increase in age is also in line with certain indications that in adults there tends to be a loss of immunity which might be accounted for by lack of reinforcement from repeated exposures. For rural populations both our own neutralization tests for immunity to poliomyelitis and Kidder's figures for immunity to diphtheria (7) indicate a slightly higher susceptibility amongst adults in general than for the 15 to 19 year age group.

munity was found to exist least often in the 1 to 5 year age group, but with increasing frequency with increase in age. In a rural population not only was the frequency of immunity in the 1 to 5 year age group less but the increase in the frequency with which it was found with increase in age was less than in the urban population. Evidence has also been presented that urban adults residing in a warm climate enjoy a degree of immunity to the disease similar to that of urban adults living in a cool climate (3). This suggests a similar widespread distribution of the virus in warm and cool climates, and in view of the greatly diminished incidence of the disease in warm climates, emphasizes the point brought out elsewhere that the occurrence of the disease may not in all circumstances parallel the distribution of the virus (4).

The results of these studies of the extent of immunity in relation to the incidence of the disease with respect to age and concentration of population when compared with similar studies on diphtheria suggest that the mechanism of dissemination of the virus and of immunization is the same as in diphtheria, the essential difference in the epidemiology of the two diseases lying in the frequency with which the "accident" of disease occurs in the process of immunization.

In infants under 1 year of age the incidence of both poliomyelitis and diphtheria is low. In the case of diphtheria the relatively low incidence in the first year of life has been shown to be due to an immunity passively derived from the mother. This passive immunity disappears in most instances before the age of 1 year is reached. Polano (8) in 1904 in testing the blood of seven mothers and the cord blood from their infants found that the amount of antitoxin was quantitatively similar or was absent in each. Von Groer and Kassowitz (8) testing mothers and infants at 143 births found antitoxin in 84 per cent of infants. In 96 per cent of these, mothers and infants corresponded with one another in having or not having antitoxin. Zingher, (8) in 1917, working with the Schick test upon 23 mothers and infants up to 3 months of age, found only a single divergent result, namely, a positive test (no antitoxin) in an infant from a Schick negative mother.

In order to determine whether the relatively low incidence of poliomyelitis in infants is similarly due to temporary maternal passive immunity we have tested the blood sera of a group of mothers together with sera from the cord blood of their newborn infants for the presence of neutralizing substance for the virus of poliomyelitis. The

sera were mixed with a virus suspension, incubated for 2 hours, placed in the ice box over night, and inoculated intracerebrally into *rhesus* monkeys in the manner described in our earlier papers (2, 3). The results of these tests are shown in Table I.

The serum of nine mothers and their infants completely neutralized the virus. The serum of one infant neutralized the virus while the animal inoculated with the mixture of virus and the mother's serum developed the disease on the seventeenth day following inoculation. The control animal in the same experiment developed the disease on the eighth day.* The serum of two mothers and their infants failed to neutralize the virus.

These results may be taken as an indication that where the mother is immune to poliomyelitis there is a passive transmission of immunity to the infant. We have not performed any tests to determine the duration of this immunity or whether or not it is fortified or prolonged by suckling, as has been shown to be the case in diphtheria. However, previous tests showing immunity in a relatively low proportion of children in the 1 to 5 year age group together with the relatively high incidence of the disease in this age group suggest that the duration of passive immunity in infants is not unlike that in diphtheria.

DISCUSSION

In this small series of tests immunity was found in a correspondingly high percentage of infants at birth and their mothers, the infants corresponding in each instance with the mother in respect to immunity with one exception, in which the result was doubtful but not contradictory. The fact that even in this small series the findings agree with what was anticipated from an analysis of the age distribution of poliomyelitis in comparison with that of diphtheria justifies the conclusion that the relative infrequency of poliomyelitis under one year of age is due in considerable measure to a passive transmission of immune bodies to the young through the placenta. No tests have been done to determine whether this passive immunity receives reinforcement

* While the result of this test is doubtful, from our previous experiments we have come to regard an incubation period of more than 14 days (using an active 7 or 8 day virus) as probably representing partial neutralization, *i.e.*, a degree of immunity.

TABLE I

Neutralization of the Virus of Poliomyelitis by Serum of Mothers and Newborn Infants

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
			cc.				
44780 (mother) 21 yrs.	49-8	515	1.0	4/ 8/30	No symptoms	+*	
44780 (baby)	48-9	515	1.0	4/ 8/30	No symptoms	+	
44798 (mother) 17 yrs.	47-6	515	1.0	4/ 8/30	No symptoms	+	
44798 (baby)	47-0	515	1.0	4/ 8/30	No symptoms	+	
44799 (mother) 19 yrs.	39-8	515	1.0	4/ 8/30	4/19 Paralyzed	−	Recovered with residual paralysis
44799 (baby)	49-5	515	1.0	4/ 8/30	4/21 Paralyzed	−	Typical microscopic lesions of poliomyelitis†
Convalescent serum No. 83	49-2	515	1.0	4/ 8/30	No symptoms	+	
Normal monkey serum	47-4	515	1.0	4/ 8/30	4/16 Paralyzed	−	Typical microscopic lesions of poliomyelitis
44803 (mother) 22 yrs.	49-9	515	1.2	4/10/30	4/17 Paralyzed	−	Typical microscopic lesions of poliomyelitis
44803 (baby)	34-5	515	1.2	4/10/30	4/18 Paralyzed	−	Typical microscopic lesions of poliomyelitis

* In the seventh column of the table *plus* indicates that the serum did neutralize the virus and *minus* that the serum failed to neutralize. *Plus and minus* indicates a doubtful or partial neutralization.

† Where *Typical microscopic lesions of poliomyelitis* appears under *Remarks*, the animals were etherized and an autopsy performed. The statement applies to examination of sections of spinal cord.

TABLE I—*Continued*

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
			cc.				
44773 (mother) 19 yrs.	51-1	515	1.2	4/10/30	No symptoms	+	
44773 (baby)	30-1	515	1.2	4/10/30	No symptoms	+	
44831 (mother) 24 yrs.	38-3	515	1.2	4/10/30	No symptoms	+	
44831 (baby)	33-2	515	1.2	4/10/30	No symptoms	+	
Convalescent serum No. 83	54-3	515	1.2	4/10/30	No symptoms	+	
Normal monkey serum	34-6	515	1.2	4/10/30	4/18 Paralyzed	−	Typical microscopic lesions of poliomyelitis
44844 (mother) 26 yrs.	54-4	515	1.2	4/22/30	No symptoms	+	
44844 (baby)	54-5	515	1.2	4/22/30	No symptoms	+	
44830 (mother) 35 yrs.	54-6	515	1.2	4/22/30	No symptoms	+	
44830 (baby)	54-7	515	1.2	4/22/30	No symptoms	+	
44845 (mother) 21 yrs.	54-8	515	1.2	4/22/30	No symptoms	+	
44845 (baby)	54-9	515	1.2	4/22/30	No symptoms	+	
Convalescent serum No. 83	55-0	515	1.2	4/22/30	No symptoms	+	

TABLE I—*Concluded*

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
			cc.				
Normal monkey serum	55-1	515	1.2	4/22/30	4/28 Paralyzed	−	Typical microscopic lesions of poliomyelitis
44860 (mother) 22 yrs.	47-0	515	1.2	5/ 8/30	No symptoms	+	
44860 (baby)	47-6	515	1.2	5/ 8/30	No symptoms	+	
44849 (mother) 23 yrs.	53-8	515	1.2	5/ 8/30	No symptoms	+	
44849 (baby)	49-8	515	1.2	5/ 8/30	No symptoms	+	
44856 (mother) 22 yrs.	49-6	515	1.2	5/ 8/30	5/25 Paralyzed	±	Typical microscopic lesions of poliomyelitis
44856 (baby)	53-6	515	1.2	5/ 8/30	No symptoms	+	
Convalescent serum No. 83	50-8	515	1.2	5/ 8/30	No symptoms	+	
Normal monkey serum	47-3	515	1.2	5/ 8/30	5/16 Paralyzed	−	Typical microscopic lesions of poliomyelitis

through transmission from the milk in suckling, nor has the duration of passive maternal immunization been determined. But the fact that eleven out of fifteen (73.5 per cent) children from 9 months to 5 years of age failed to neutralize the virus (2) (two of them were 9 months and 11 months old respectively) together with the fact that as in the case of diphtheria the incidence rises sharply after 6 months of age, suggests the probability that the mechanism of maternal immunization to poliomyelitis is similar to that of diphtheria.

At this point we may discuss briefly the results of these and previous tests for immunity to poliomyelitis from the point of view of their statistical significance. The tests performed on individuals of any one category is small—too small, as we have repeatedly pointed out, to establish the point in question when taken alone. But even where samples are small, if we observe several such samples and find them consistent with each other and with other comparable observations, the statistical significance of such observations rises rapidly.

In the first place, as discussed in detail in our earlier communications regarding the neutralization test, the test may be considered as a reliable indication of immunity.

The idea that the majority of urban adults have become immunized to the virus of poliomyelitis, upon which our present conception of the

TABLE II

Summary of Neutralization Tests for Virus of Poliomyelitis—Urban Adults

	Immune	Per cent	Not immune	Per cent
Northern adults	7	85.7	1	14.3
Southern adults	19	90.5	2	9.5
Northern mothers	10	83.3	2	16.7
Total urban adults	36	87.8	5	12.2

epidemiology of the disease hinges, is not only supported by tests which show immunity in increasing frequency up to adult age but by three separate sets of tests on urban adults themselves, all of which, as shown in Table II, are in close agreement.

The curves for immunity in normal individuals at different age groups both in urban and in rural populations have a similar trend. The diminished extent of immunity in a rural population is in accordance with diminishing chances of contact transmission of the virus, due to decrease in density of population.

The extent of immunity at different ages and in urban and rural populations, as well as in mothers and infants, is in accord with what might be expected from an analysis of the age distribution of the disease.

Finally, the extent of immunity to poliomyelitis in each age group

tested corresponds closely with that of immunity to diphtheria. This with other evidence that the process of immunization is essentially the same for the two diseases is regarded as additional support for the statistical validity of the relatively small number of observations which have been made on poliomyelitis.

SUMMARY

Neutralization tests for the virus of poliomyelitis on blood serum of urban mothers and their newborn infants showed that immunity was present in ten out of twelve (83 per cent) infants and in ten out of twelve (83 per cent) mothers, with a complete correspondence between mother and infant. These tests point to passive transmission of immunity from mother to infant. Previous tests on other children (1 to 5 years) indicate that immunity in infants is transitory. Previous observations concerning the extent of immunity in urban adults are confirmed and extended. The results of these tests are in accord with the age distribution of poliomyelitis and parallel corresponding observations in diphtheria.

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CULTIVATION OF VACCINE VIRUS

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(Received for publication, June 21, 1930)

Many investigators have cultivated vaccine virus in bits of living tissue embedded in plasma. These small cultures were made on coverslips which were then inverted and sealed over hollow ground slides. For many reasons methods of making larger cultures are highly desirable. To this end, Carrel and Rivers (1) devised a procedure whereby cultures of vaccine virus were made in 2 to 8 cc. amounts. This was accomplished by inoculating minced chick embryo tissue with vaccine virus and then embedding it in diluted chicken plasma in Carrel flasks. This method was improved upon by Maitland and Maitland (2) who used a fluid medium consisting of minced chicken kidney suspended in chicken serum diluted with Tyrode's solution. Later Muckenfuss and Rivers (3) altered Maitland's medium by substituting rabbit testis and serum for chicken kidney and serum. The fact that certain viruses, *e.g.*, vaccine virus, Virus III, herpetic virus, will multiply in a medium of this nature has been amply verified by the work of Eagles and McClean (4), Andrewes (5–7), and Maitland and Laing (8).

The work to be described at this time has to do with the simplification of the technique for the cultivation of vaccine virus suitable for Jennerian prophylaxis in man. A medium, in which tissues and serum from halfgrown or adult animals are used, may easily permit the entry of an unknown virus into the cultures as a contaminant. The chance of such an occurrence can be lessened by the use of minced chick embryo suspended in Tyrode's solution. The possibility that such a medium would be appropriate was suggested by an experiment in which Andrewes (6) showed that Virus III survived and formed inclusions in rabbit testicular tissue suspended in Tyrode's solution.

Methods and Materials

Virus.—Levaditi neurovaccine virus that had been propagated for 6 months in rabbit testicles was used to initiate the cultures.

Tissues.—Testicular tissue was obtained aseptically from rabbits, washed in Tyrode's solution, placed in a sterile watchglass contained in a Petri dish, minced with scissors, and then distributed in proper amounts into flasks by means of a pipette. Embryo tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (1) or that of Eagles and McClean (4).

Fluids.—When serum was used it was collected from rabbits, and diluted with 3 parts of Tyrode's solution. Tyrode's solution, pH about 7.2, prepared according to the following formula was sterilized by filtration: NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; $NaHPO_4$, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc.

Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. At first, cultures were made in Carrel D flasks. Then 50 cc. Erlenmeyer flasks, stoppered with cotton over which two layers of tinfoil were placed, were used. Neither type of container was found entirely satisfactory for the work. Finally a flask was designed (collar flask) which proved to be suitable. The mouth of the flask (Text-fig. 1) is of sufficient size to permit the removal of the contents by means of a pipette. It is closed by a cotton plug over which are stretched two or three layers of tinfoil. The enlargement in the neck reduces the amount of water of condensation on or near the cotton plug and also prevents the water that does touch the cotton from running down and contaminating the culture. The small opening, plugged with cotton, on the side serves for ventilation. The bottom is perfectly flat, thus providing a large area over which a thin layer of culture may be evenly distributed. The flasks are made in two sizes. The dimensions of the large one are: height, 14 cm., diameter of mouth, 3.5 cm., diameter of base, 9 cm. The measurements of the small one are: height, 9 cm., diameter of mouth, 2.5 cm., diameter of base, 4.5 cm. In the large flasks, 2 to 4 gm. of tissue suspended in 15 cc. of Tyrode's solution were used, while in the small ones 0.5 to 1 gm. of tissue suspended in 5 cc. of Tyrode's solution was employed.

Preparation of Cultures.—Approximately 1 gm. of tissue was used to each 5 cc. of diluted serum or Tyrode's solution. Testicular or chick embryo tissue suspended in its vehicle was distributed in flasks. Prior to use, the sterility of the medium was tested on blood agar and in broth. During this interval (48 hours), the prepared flasks were stored at +5°C. The medium was then inoculated with 0.1 to 0.2 cc. of virus emulsion, and the cultures were incubated at 37°C. for 7 days. New cultures were made by the direct transfer of a few drops of the old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations.

Titration of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution was injected into the shaved skin of rabbits. Daily observations of the animals were made for a week.

EXPERIMENTAL

In our investigation concerning the cultivation of vaccine virus, many experiments have been performed. All of them will not be

TEXT-FIG. 1. Type of flask used for the cultivation of vaccine virus

described, inasmuch as some are similar to experiments made and reported by others (8). Part of our work, however, is different from that previously described and the results are summarized in Chart 1.

The first cultures of Series A (Chart 1) were made February 17, 1930. A medium consisting of rabbit testicular tissue and a mixture of rabbit serum (1 part) and Tyrode's solution (3 parts) was inoculated with

vaccine virus and then distributed in Carrel D flasks. Virus from a culture of the third generation of Series A was inoculated into a flask (Series B) of medium comprised of chick embryo tissue suspended in

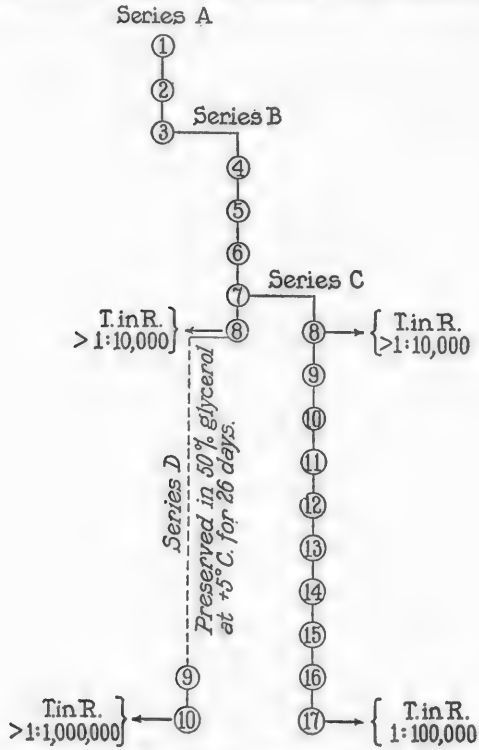

CHART 1. Summary of experiments on cultivation of vaccine virus.

The media used in the different series were as follows: Series A: Rabbit testicular tissue in a mixture of rabbit serum and Tyrode's solution. Series B: Chick embryo tissue in a mixture of rabbit serum and Tyrode's solution. Series C and D: Chick embryo tissue in Tyrode's solution.

T. in R. indicates titer in rabbit.

rabbit serum (1 part) and Tyrode's solution (3 parts). Seventh generation virus (Series B) was inoculated into a medium of chick embryo tissue suspended in Tyrode's solution (Series C). At this time collar flasks were first employed and since then they have been in general use.

The virus in Series C has been propagated for 10 generations, and is still under cultivation.

The fact that the virus multiplied is obvious and a mathematical proof seems superfluous. Nevertheless, one estimation will be given. The titer of the eighth generation virus (Series B) was >1:10,000. This virus mixed with glycerol was stored at +5°C. for 26 days. Then the preserved virus was diluted 1:100 with Tyrode's solution, and 5 drops of the dilution were inoculated into 5 cc. of fresh medium. After a 9-day period of incubation, 4 drops of this culture were added to 15 cc. of fresh medium. After incubation the titer of the last culture was 1:1,000,000 (Series D).

From the work summarized in Chart 1, it is obvious that the virus can be transferred without difficulty from one kind of medium to another. Furthermore, culture virus, mixed with glycerol, sealed, and stored at +5°C., can be preserved for a long time. In this manner virus has been preserved for 64 days without great loss in potency. Moreover, the preserved virus can be employed as an inoculum for the initiation of fresh cultures.

The virus propagated in cultures for a long time seems to have lost none of its characteristics, for, when it is spread on the scarified shaved skin of a rabbit, typical vaccinal lesions occur. Moreover, serum from a rabbit immune to Levaditi's neurovaccine virus completely neutralizes the culture virus. Finally, an immune serum prepared with the culture virus neutralizes the Levaditi vaccine virus.

DISCUSSION

A simple method for the *in vitro* cultivation of vaccine virus has been devised. The flasks employed are easy to manipulate and permit the production of large amounts of virus. Moreover, the medium is innocuous and inexpensive, consisting of minced chick embryo suspended in Tyrode's solution. In spite of the simplicity of the procedure, however, one should not forget the fact that living susceptible host cells are employed. No one has as yet been able to demonstrate that vaccine virus can multiply in the absence of such cells.

The facility with which vaccine virus can be handled and cultivated *in vitro* varies with the strain. This fact has been evidenced by previous experiences in our laboratory and also in that of Eagles and

McClean (4). In the work reported at this time a neurovaccine virus was employed. Studies have been planned to determine whether it is possible to adapt a dermovirus to the type of medium used by us.

SUMMARY

1. A strain of neurovaccine virus was cultivated in a medium consisting of minced chick embryo suspended in Tyrode's solution.

2. The virus upon cultivation apparently lost none of its essential characteristics.

3. The culture virus can be preserved and stored for long periods of time. Furthermore, the preserved virus can be used to initiate fresh cultures.

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THE STANDARDIZATION OF ANTIPNEUMOCOCCIC SERUM TYPES I AND II

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(Received for publication, June 17, 1930)

The mouse protection test for establishing the value of antipneumococcic serum does not give consistent results unless a large number of mice are used. Friedlander, Sobotka and Banzhaf (1) used the specific polysaccharide of the pneumococcus as an antigen for precipitation tests, to determine the potency of serum. Their findings agreed fairly well with the mouse protection test of Felton (2).

In confirming the work of Heidelberger and Avery (3), on the pneumococcus polysaccharide, we were struck by the consistent results obtained in successive precipitation tests with any one serum. At the same time, we have long been aware of the difficulty in the interpretation of the mouse protection test in judging the potency of antipneumococcic serum. We, therefore, have undertaken parallel studies on a series of antipneumococcic serums, using both the specific polysaccharide precipitation and mouse protection tests. A close agreement between the results of the two methods was found to exist.

The difficulty in the preparation of polysaccharide of the high purity obtained by Avery and Heidelberger would be a barrier to the practical routine application of the polysaccharide precipitation test. We found, however, in comparing different lots of polysaccharides of varying purity, that results were comparable with each lot.

Crude polysaccharide prepared by the following technique was found very satisfactory for use as antigen in the precipitation test:

An 8 day broth culture of the type pneumococcus is evaporated to about one-fifteenth the original volume. The material is chilled and trichloracetic acid crystals added to make a 5 per cent solution. A precipitate is formed and removed by centrifugation as soon as possible. To the clear supernatant about one and one-half volumes of alcohol are added and centrifuged. The alcoholic pre-

cipitate contains the polysaccharide which is soluble in water and is purified by repeated re-precipitations with alcohol. The final precipitation is usually done with acetone. Further purification does not seem necessary.

Polysaccharide made by this method when dried and dissolved in saline 1 to 10,000 can be satisfactorily used for antigen in the precipitation test.

The technique of the precipitation test used by us is as follows: Equal volumes of serum dilutions and a constant polysaccharide dilution (1 to 10,000) are mixed and placed in a water bath at 37°C. The water level should be not more than one-half that of the test mixture. The test is read after 2 hours. Further precipitation in higher dilutions may occur after longer incubation at 37°C., or a subsequent period on ice, but the result is not as clear cut, variations being more frequent than when the 2 hour reading is used.

The interpretation of the test is based on a comparison of the titer of the complete precipitation of a standard serum, F-146 (supplied by Felton), as containing 200 protective units* per cubic centimeter for Pneumococcus Types I and II, with that of the unknown test serum.

In repeated tests, using a solution of Type I polysaccharide of 1 to 10,000, the standard serum gave complete precipitation in a dilution of 1 to 20. On this basis the number of units for Type I of the test serum would be expressed by the following equation.

$$\frac{\text{Dilution of test serum}}{20} \times 200 = \text{Number of units of the test serum.}$$

The results of comparative mouse protection and polysaccharide precipitation tests with a portion of the many polyvalent antipneumococcic Type I and II serums, tested, are shown in Table I. Only results of tests with Type I are given. Type II values can also be tested by the polysaccharide precipitation test, the equation of calculation, however, being different as the standard serum F-146 with a protective unit value of 200 units in Type II, results in a complete precipitation at a dilution of 1:35 with the specific Type II polysaccharide used by us.

* The standard value of this serum has recently been changed by Felton after testing with the different strains of pneumococcus used by the various laboratories, to 500 units per cubic centimeter Type I and Type II.

The interpretation of the mouse protection by direct calculation for unit value according to virulence is frequently difficult. The variations and irregularities in the time of the deaths frequently make necessary a repetition of the test before results are obtained which satisfactorily check with the established value of the standard serum.

TABLE I

Type I Antipneumococcus Protective Units Estimated by the Mouse Protection and Polysaccharide Precipitation Tests

Serum	Mouse test	SSS Ppt.
F-146	200	200
7892-N	100	120
7879-A	80	80
7895-G	200	220
7899-M	100	100
7900-H	50	80
7905-I	300	300
7908-I	300	300
8081-A	40	50
8082-A	50	50
MM8843-A	600	560
MM8843-B	100	90
MM8843-C	50	20
46130	50	50
48398	400	350

* "SSS Ppt." is the designation used by Heidelberger and Avery for the "soluble specific substance"—polysaccharide responsible for the precipitate.

The unit values given in Table I are therefore calculated by direct comparison with the 200 unit standard serum F-146.

Table II shows the results obtained with the polysaccharide precipitation and mouse protection test, on using serum from consecutive bleedings from horses under immunization. The protection test used in this series was that endorsed by the Hygienic Laboratory and controlled by their standard serum. The serums are classified as *good*, *fair* or *poor*, according to the degree of protection, expressed in percentage, obtained in the group of mice placed on test. The ease with which one can be misled by the use of the mouse test alone is shown. For example in Serum 7892 the value of the bleeding of 2–15–30 and of

3–15–30 is more accurately determined by the polysaccharide precipitation test. The results of tests on bleedings which prove horses to be producing serum of a higher value—such as Serum 7905—are consistent with the mouse protection test. The exact status of serum from horses on the borderline of productivity is much more accurately

TABLE II

Antipneumococcic Type I

Protective Units in Consecutive Horse Bleedings

Date	SSS Ppt.	Mouse	Date	SSS Ppt.	Mouse
Serum 7879			*Serum 8083*		
1–28–30	90	Fair	12–26–29	180	Fair
2– 1–30	80	Good	1–26–30	100	Fair
2–15–30	50	Fair	2–10–30	150	Fair
3– 7–30	30	Poor	3– 7–30	80	Fair
3–31–30	30	Fair	3–15–30	100	Fair
			4– 1–30	80	Fair
Serum 7892			*Serum 8110*		
1–30–30	180	Good	12– 4–30	—	Poor
2–15–30	220	Fair	1–30–30	240	Good
3– 1–30	100	Fair	2–15–30	180	Good
3–15–30	90	Fair	3– 7–30	30	Fair
			3–22–30	50	Fair
Serum 7905			*Serum 8113*		
1–15–30	200	Good	9–25–29	—	Poor
1–30–30	300	Good	12– 4–29	—	Fair
2–15–30	280	Good	1–30–30	180	Good
3– 1–30	280	Good	2–15–30	250	Fair
			3– 1–30	280	Good

determined by the polysaccharide precipitin test, as shown in bleedings of Serums 8083, 8110 and 8113.

At present the immunization of all horses used in the production of antipneumococcic serum is followed by means of the precipitation test. Mixtures of bleedings for concentration or other purposes, are checked with the mouse protection test.

DISCUSSION

The standardization of antipneumococcus serum Types I and II has been a very difficult problem on account of the variable results obtained with the mouse protection test. The difference in the virulence of the cultures used, of Pneumococcus Types I and II makes comparison of the mouse tests of various laboratories impossible. Even within one laboratory, in which the same strain is always used, and with every care for absolute duplication of method and accuracy of technique, variations occur which often make the interpretation of the test difficult, and costly repetition necessary. This is due to the very nature of the test, susceptibility of the mouse to a virulent pneumococcus being so marked that the smallest deviation in the number of organisms injected into the test animal leads to most confusing irregularity in the time of death. Also variation in number of organisms is unavoidable by the method because of the procedure of making dilutions as a basis for dosage.

We have found that the precipitation test is practical and gives comparable results on the repetition of tests. Similar conclusions have been reached by Heidelberger, Sia, and Kendall (see the following paper (4)).

It seems highly probable that in the case of organisms elaborating specific polysaccharides this method of testing can be used for the standardization of the antiserums. We have succeeded with anti-anthrax serum, the standardization of which has been very unsatisfactory, and we are proceeding with tests of other antibacterial sera.

CONCLUSIONS

1. The mouse protection test used for the standardization of antipneumococcic serum Type I and II is very unreliable.

2. The specific polysaccharide precipitation test can be used to establish the comparative value of a test serum to a standardized serum.

3. The polysaccharide used for such tests does not have to be of the highest purity.

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SPECIFIC PRECIPITATION AND MOUSE PROTECTION IN TYPE I ANTIPNEUMOCOCCUS SERA*

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(Received for publication, June 17, 1930)

Specific precipitation has long been known to occur in antipneumococcus sera showing marked protective power in mice (1), but it has been possible only recently to obtain indications of a parallel variation in these two properties. Thus Friedlander, Sobotka and Banzhaf (2) showed that the number of mouse protection units in certain Type I and Type II antipneumococcus sera varied in the same sense as the "precipitin index." In the preceding communication Zozaya, Boyer, and Clark (3) have shown that it is possible by means of the precipitin test to obtain within 2 hours numbers which agree excellently with the mouse protection units found in fifteen sera.

The great advantages of a rapid *in vitro* test for the potency of antipneumococcus sera over the tedious, expensive, and often uncertain mouse protection test had led the writers to search for a possible relation between specific precipitation and mouse protection in Type I antipneumococcus sera on the basis of their recent quantitative study of the reaction between the specific polysaccharide of Type III pneumococcus and its homologous purified antibody (4) as well as on the basis of almost completed data on the corresponding Type I reaction. While this work has not resulted in so simple and rapid a routine test as that of Zozaya, Boyer, and Clark, the writers feel that it establishes on an experimentally verified and theoretically reasonable basis a definite parallel between maximum specific precipitation in Type I

* The work described in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York City.

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antipneumococcus sera and mouse protection. Furthermore, this parallel can be drawn with sufficient exactness to warrant the statement that in the group of widely varying sera studied under the definite empirical conditions described below, sera containing 7 mg. or more of specifically precipitable protein per cubic centimeter showed a mouse protection value of 1000 units and over. It is suggested that should these, or similar values be found by other workers to hold for sera at their disposal, a quantitative grading of Type I antipneumococcus sera on the basis of their content of specifically precipitable protein would not only be feasible, but would also be far quicker, cheaper, and more susceptible of duplication in other laboratories than the present generally accepted mouse protection values.

EXPERIMENTAL

1. Type I Pneumococcus Specific Polysaccharide.—The Type I "soluble specific substance" used was prepared as in a preceding paper (5). Since an ash-free preparation of highest purity is not essential, an abbreviated method is here given.

20 liters of 5-day Type I pneumococcus culture in meat infusion phosphate broth containing 0.3 per cent of glucose are autoclaved and concentrated on the water bath to a volume of 1 liter. The concentrate is precipitated with 1.4 liters of alcohol and put through the initial 3-layer separation (6). The middle layer is treated with 200 to 250 cc. of hot water until all lumps are dissolved, and the mixture is centrifuged and the precipitate washed twice with small amounts of water. The clear solution and washings are chilled and acidified strongly to Congo red with 1:1 hydrochloric acid, centrifuging off in the cold and washing with a little 0.01 normal hydrochloric acid any precipitate formed at this point. The solution, the volume of which should not exceed 500 cc., is stirred vigorously, precipitated with 1.5 liters of chilled alcohol, and allowed to stand in the cold over night. The precipitate, after centrifuging, is taken up in water, with the addition of enough sodium hydroxide to maintain alkalinity. Water is added until all of the specific substance is in solution, and the remaining trace of insoluble material is centrifuged off. The supernatant, at a volume of 100 to 125 cc., is treated with 10 gm. of sodium acetate, and when this has dissolved, is chilled, precipitated with 70 cc. of cold alcohol, allowed to stand in the cold over night, and centrifuged in the cold. The precipitate is redissolved in 200 cc. of water, the solution is centrifuged, and the supernatant acidified with glacial acetic acid to pH 3.4, at which point the specific polysaccharide flocculates readily and is centrifuged off.

The supernatant should contain very little specific substance, but if it does, the pH should be adjusted in the direction of further flocculation and the precipitates combined. The specific substance is taken up in 100 cc. of chilled 0.05 normal acetic acid, centrifuged off again, and washed in the centrifuge bottle, first with redistilled alcohol and finally with redistilled acetone. It is then filtered on a Buchner funnel, washed with redistilled acetone, and dried *in vacuo* at room temperature. The yield in a typical instance was 0.8 gm.,* containing 0.5 per cent ash. Other analytical data, calculated to the ash-free basis, were: $[\alpha]_D$, + 299°, N, 5.0 per cent, values practically identical with those of the best preparations previously reported (5). If larger amounts of broth are worked up it is best to repeat each precipitation.

2. Titration of Sera by the Precipitin Method.—If, on addition of a 1:10,000 saline solution of the specific polysaccharide to a portion of the serum, precipitation is rapid and heavy, 0.5 cc. of the serum should be used for the quantitative determination. This will suffice for all except very low grade sera, of which it is better to use 1 cc. The sera should be measured in duplicate with accurately calibrated pipettes into wide agglutination tubes (dimensions of 10 x 75 mm. have been found suitable). If 0.5 cc. serum has been used, 1 cc. saline is then added to each tube, followed by 0.5 cc. of a saline solution containing 1 mg. of the specific polysaccharide per cubic centimeter, to make a total volume of 2 cc. Ordinary uncalibrated pipettes are adequate for the saline and polysaccharide. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the finger tips rapidly and repeatedly diagonally down the side of the tube. After the tubes have been allowed to stand 2 hours in the water bath at 37° and over night in the icebox,† the plugs are removed and the tubes centrifuged for 10 minutes at 1000 revolutions per minute, either in a refrigerating centrifuge‡ or immersed in ice-water. The supernatant liquid is then carefully drained off by inverting the tube and wiping the mouth of the tube with filter paper after a few minutes. The tubes are then placed in ice-water and the precipitates are each washed with 2 cc. of an ice-cold 1:20,000 saline solution of the specific polysaccharide, mixing the contents as before. It appears to make little difference whether or not the disc of precipitate is loosened from the bottom of the tube. After ½ hour in the cold the tubes are again centrifuged and drained

* Since the isoelectric substance is insoluble, solutions must be made up with the aid of alkali.

† Sterile technique should be employed up to this point. In the case of low grade sera identical values are obtained if the tubes are allowed to stand in the water bath only ½ hour, followed by ½ hour in ice-water before proceeding. In more potent sera results a few tenths of a milligram per cubic centimeter too low are obtained by shortening the process in this way.

‡ Supplied by the International Equipment Co., Boston, Massachusetts.

as before. About 0.5 cc. of water is then added to each tube, shaking as before until the precipitate is loosened, after which the disc is dissolved by the addition of 2 drops of normal sodium hydroxide solution with rotation of the tube until the precipitate has disappeared. If the disc is allowed to stick to the glass, solution is much slower. The solution is then rinsed quantitatively into a micro-Kjeldahl flask or tube and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found $\times$ 6.25 = specifically precipitated protein.

3. Mouse Protection Tests.—For the biological estimation of the amount of protective antibodies contained in various samples of Type I antipneumococcus sera, the method described by Felton (7) was employed. According to this method, a *unit* (of antipneumococcus protective antibody) is defined as "that fraction of a cc. of serum which will protect against one million fatal doses of an 18-hour serum broth culture. The culture used must be of such virulence that 3 to 10 organisms when injected intraperitoneally into a mouse kill the mouse in from 36 to 48 hours."

Briefly, the method consists of the intraperitoneal injection into white mice of 0.5 cc. of a 1:200 dilution* of an 18-hour serum broth culture of the Type I pneumococcus mixed with an equal amount of the dilution of the Type I antipneumococcus serum to be tested. The immune serum dilutions were made in sterile normal saline and the dilutions used were 1:25, 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, 1:1600, no attempts being made to titrate more closely by the use of intermediate dilutions. The organism and the immune serum dilutions were taken up into a syringe and injected as soon as possible intraperitoneally into mice. For each serum dilution tested, mice were injected in triplicate, and observed for 96 hours. The highest immune serum dilution protecting two out of the three mice for a period of 96 hours was taken as the protective unitage of the serum.

The results obtained are given in Table I.

DISCUSSION

In the course of a quantitative study of the precipitin reaction between Type I pneumococcus specific polysaccharide and purified Type I pneumococcus antibody, it was found that maximum precipitation occurred when the initial concentration of specific polysac-

* Calculated to contain 500,000 lethal doses.

TABLE I

Specific Precipitation and Mouse Protection in Type I Antipneumococcus Sera

Sample No.	Source	Agglutination titer	Units found in individual mouse protection tests	Individual determinations (in duplicate) of sp. pptble. protein	Average mouse protection units	Average specifically pptble. protein
				mg. per cc.		*mg. per cc.*
B108C	4*	1:10	50	0.7 0.6	50	0.7
101	1 (diagnostic)	1:60	100 100 100	1.7 1.7 1.7	100	1.7
110	5 (standard)**		200	1.9	200	1.9
108B	4*	1:40	200	2.0 2.0	200	2.0
105	3 (diagnostic)	1:60	800 400 800 400 600†	3.1 3.3 3.4	600	3.3
108A	4*	1:40	600	3.8 3.8	600	3.8
107	3 (diagnostic)		800	5.0 4.8	800	4.9
103	3 (therapeutic)	1:120	1200 1000 1000 800	6.9 6.5 6.7 6.6	1000	6.7
104	2 (diagnostic)	1:160	>1200‡ 1200 >1600‡ 800	7.0 6.9 7.4 7.1 6.9	1200	7.1
102	1 (diagnostic)	>1:120	>600‡ >800‡ 1200	7.4 7.9 7.6	1200	7.6
106	3 (therapeutic)	1:160	>1200‡ >1600‡	9.7	1600	9.7

* Number 8843 in the paper by Zozaya, Boyer and Clark.

** Number F 146 in the paper by Zozaya, Boyer and Clark.

† This dilution was not used until the first four results had been obtained.

‡ Highest dilution tested.

charide was 1:2,000. Since, however, all but a small fraction of the specifically precipitated protein is carried down at one-half this concentration, the latter strength is used in the test herein given, in the interest of economy of specific substance. Similarly, the precipitate is washed with a 1:20,000 solution of the polysaccharide as this concentration is adequate to prevent appreciable dissociation of the precipitate and the nitrogen content of a solution of this concentration is too small to interfere.

It can not be claimed that the nitrogen figures obtained are more than an approximate representation of the specifically precipitable protein in the sera tested, since a single washing of the compact disc-like precipitate would scarcely remove all entrained inert serum proteins. On the other hand, washing with 1:20,000 polysaccharide solution, as is done in the test, probably fails to prevent absolutely dissociation of the precipitate formed under optimum conditions, so that these two errors tend to balance each other. Since duplicates and repeated determinations usually check, at least consistent results are readily obtained. Moreover, any number of sera may be tested at a time.

From Table I it will be seen that the sera tested, when arranged in the order of increasing specifically precipitable protein per cubic centimeter, are also found to be in the order of increasing mouse protection units. By the methods employed, 7 mg. of specifically precipitable protein per cubic centimeter corresponded roughly with a mouse protection value of 1000 units, and it is felt that an easily reproducible and readily determinable standard system of grading could be worked out along these lines.

SUMMARY

1. A rapid and simple method is given for the approximate determination of the specifically precipitable protein in Type I antipneumococcus sera.

2. It is shown that a close parallel exists between the specifically precipitable protein and the number of mouse protection units in a wide variety of Type I antipneumococcus sera.

3. Owing to the consistent results obtained and the rapidity, simplicity, and economy of the method, its use is proposed instead of the

mouse protection test as a basis for the titration of standard sera and the comparison of others with a standard.

4. A method is given for conveniently preparing highly purified specific polysaccharide of Type I pneumococcus.

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THE EFFECT OF INFLAMMATORY REACTIONS ON TISSUE IMMUNITY

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Plates 18 and 19

(Received for publication, June 6, 1930)

The problems of immunity in the past have been approached chiefly by noting alterations in the blood plasma. Although it has long been recognized that the invaded tissues also play a significant rôle in resistance, the difficulties encountered in studying this mechanism have greatly retarded a complete understanding of the problem. Most of the studies on cellular immunity have been devoted to various aspects of tuberculosis, for in this disease true humoral immunity is strikingly absent. Only recently has it been adequately appreciated that most "normal individuals" harbor a variety of pathogenic organisms in chronic foci in the naso-pharynx or elsewhere in the body and derive from them a generalized alteration of tissue response quite analogous to that found in tuberculosis (1). This bacterial allergy is usually demonstrated by injecting small amounts of broth filtrates of the organism intradermally and noting the degree of redness and infiltration that has developed 24 hours later. The skin reactions are not type specific and may be demonstrated for many organisms biologically unrelated. The relationship of tissue reactivity to bacterial invasion and the development of disease is obviously difficult to study in the human being. Consequently we have transferred our investigations to rabbits using as our infecting agent *B. lepisepticum* which is harbored in the naso-pharynx and is the cause of the majority of acute and chronic lesions of the respiratory tract in this convenient laboratory animal (2, 3, and 4). Infections of the skin chiefly have been employed because here it is easiest to observe the progress of the lesion.

Before undertaking any experiments it has been our routine procedure to skin-test the animals with 0.2 cc. of a 48-hour broth filtrate of *B. lepisepticum*. Readings were made 24 hours later and the animals classified, according to the size, redness and induration of the injected skin, into the following groups: (1) "negative reactors," (2) "weak reactors," (3) "moderate reactors" and (4) "strong reactors." This classification has proved important because the strong reactors show a definite tendency to localize the infection, even when the serum in such animals (in those animals tested) shows no agglutinins or demonstrable protective substances against the organism used.

TABLE I

Comparative Infections in Strong Reactors and Weak Reactors

	Total infection mortality	Died		Survived	
		Large lesion	Small lesion	Large lesion	Small lesion
Strong reactors.	9	7	2	5	13
Total number, 27	(33.3%)	(25.8%)	(7.4%)	(18.5%)	(48.1%)
Weak reactors	40	34	6	3	10
Total number, 53	(75.5%)	(64.1%)	(11.3%)	(5.6%)	(18.8%)

Contrasted Infections in Weak and Strong Reactors

The negative and weak reactors, unless serological immunity is present, show but slight ability to limit the spread of the local lesion. After the injection of about 0.1 cc. of a light suspension of a virulent strain of *B. lepisepticum* (R. D.) into the skin of the flank, the characteristic infection begins as a small, pale papule at the site of inoculation and is well developed in 24 hours. Hemorrhagic necrosis appears quickly and from its intensity may be predicted the severity of the infection which will subsequently develop. Within from 5 to 10 days the lesion spreads downward over the abdomen forming a large, black oedematous mass. The animal becomes feverish and frequently dies. Post mortem examination usually reveals a positive blood culture but seldom metastatic lesions.

The strong reactors may occasionally develop similar large lesions

and succumb to the infection; in such cases they quickly lose all skin reactivity, hemorrhagic necrosis appears and the progress of the disease is in every way identical to that in the poor reactors. More frequently, however, the site of the infection is larger and redder after the first 24 hours and spreads but little thereafter. Histologically, the typical lesions of the strong reactors show more leucocytes, both of the polymorphonuclear and monocytic variety, and much less cellular disintegration than those of the weak reactors.

In Table I is shown the outcome of comparable infections in a series of eighty rabbits. It is obvious that there is a higher degree of resistance among the strongly reacting groups, this immunity being manifested chiefly by lesions more limited in extent. It might be argued that the presence of allergy presupposes a past "infection experience" from which a generalized immunity might also be derived. Evidence for this cannot be demonstrated in the serum of these animals. It therefore appears that the immunity furnished is *cellular* in nature and the present work is reported in an attempt to analyze some of the factors in such a type of immunity.

Infection of Skin Areas Previously Infiltrated with Bacterial Filtrates

Much study has been devoted to the fate of various pathogenic bacteria when injected into inflamed tissues. The results reported depend upon the organisms used, the irritant employed, and the duration and intensity of the inflammation before the infecting agent is introduced. An excellent summary of the important work on this subject has recently been made by Opie (5).

Besredka (6) observed that filtrates of certain bacteria applied to the skin rendered the skin immune to these bacteria and suggested the use of these filtrates therapeutically. Gay (7) and his co-workers have shown that a non-specific sterile inflammation which caused an increase of clasmatocytes protected the pleura of rabbits against streptococcus. Rivers and Tillett (8) and Mallory and Marble (9) have demonstrated a local protection of the skin to streptococcus and staphylococcus by infiltrating the dermis 24 hours previously with bacterial filtrates, or even plain broth, which suggests very strongly that there is no specificity to the phenomenon.

We have been able to demonstrate a local protection of the skin to virulent *B. lepisepticum* by infiltrating 24 hours previously with

filtrates of various strains of this organism. Equal protection, however, was obtained with filtrates of *B. coli*, *Streptococcus hemolyticus* and to a less extent with plain broth. When filtrates and organisms are injected into the tissues simultaneously, there is no demonstrable protection.

Normal rabbits showing weak skin reactions, and presumably susceptible to infection, were shaved over the flanks at least 48 hours before the experiments. An area of skin about 4 cm. in diameter was infiltrated with one of the above filtrates by injecting small amounts at adjacent points. The total quantity employed was usually 0.7 cc. 24 hours later slight redness and oedema were present depending upon the reactivity of the skin of the individual animal. Skin thus prepared was injected with 0.1 cc. of standard virulent strain of *B. lepisepticum* (R. D.). In some animals a similar injection was made in the opposite flank.

TABLE II

Effect of Infiltrating Skin with Bacterial Filtrates 24 Hours before Inoculation with Virulent B. lepisepticum

Total number of rabbits, 26	No necrosis Infection localized to filtrate treated skin	9	34.7%
	No necrosis in filtrate treated area Infection spread beyond filtrate area with usual necrosis	12	45.7%
	Necrosis present in filtrate treated skin	5	19.2%

In others no control injection was made in order to avoid the possible inhibiting effect of another lesion. Many of this group were infected intracutaneously several days later to demonstrate absence of generalized immunity.

The results summarized in Table II indicate roughly three different types of response:

1. A complete localization of the lesion to the infiltrated skin.

2. Spreading of the lesion with development of the usual large necrotic area over the abdomen, *but in which the skin showed no necrosis in the filtrate-treated portion.*

3. Lesions similar to the above (2) except for necrosis in the filtrate-treated portion. This necrosis was usually very slight except in one case. This animal received only broth infiltration before being injected.

Infection in the prepared skin often produces, within a few hours, a diffuse, red lesion in which the absence of necrosis is very striking and in which healing takes place more promptly than in control infections. This absence of necrosis was also noted by Mallory and Marble and appears to the writer to be of great significance, for we can assume that the inflammation surrounding a lesion is due to the diffusion of bacterial products resembling those found in filtrates. A non-specific mechanism for localizing certain infections might be assumed to exist if this zone of preparation antedates the bacterial invasion by sufficient time or if the animal possesses cells capable of rapid stimulation by these substances, as is the case in the allergic group.

Infection of Skin Areas into Which B. lepisepticum Immune Serum Has Been Infiltrated

The type of resistance just described must not be confused with that conferred upon an area of skin by injecting previously or simultaneously with the organisms a small amount of immune serum. We have employed for our experiments a stock mixture of sera obtained from rabbits convalescing from a large cutaneous infection caused by *B. lepisepticum*. This serum contains practically no agglutinins for the organism, but protests in minute amounts an area of skin infiltrated with it several hours before infection. In such cases the site of inoculation is hardly visible. When organisms and immune serum are injected simultaneously there is often a redness and induration at the site of injection which tends to heal rapidly and never develops into a formidable lesion, even in the most susceptible animal. In the last analysis the efficacy of immune serum in *B. lepisepticum* infection is its ability to protect the tissue cells of all varieties from bacteria and their injurious products.

Infection of Skin Areas Reacting to Various Chemical Irritants

Before analyzing the various factors involved in the localization of infection in animals showing no serum immune substances, it is important to contrast the effect of filtrates described above with that of various chemical irritants such as dilute acetic acid, croton oil and xylol.

Normal weak reactors were injected intradermally with small amounts of one of these substances. Within 24 hours a large red lesion developed with varying degrees of necrosis at the point of injection and often with puffy oedema over the dependent portions. Bacteria injected into such an area spread rapidly, produc-

TABLE III

Infection of Areas of Skin Inflamed by Chemical Irritants 24 Hours Previously

Animal number	Chemical irritant used	Amount of irritant	Severity of local inflammation	Result of infection of local inflamed area after 24 hours	Result of infection of a control area of normal skin after 24 hours
		cc.			
3-42	Acetic acid 2%	0.5	Severe	Very large, necrotic lesion	Not done
3-43	Xylol	0.5	Severe localized	Large, necrotic, spreading	Not done
3-53	Croton oil	0.1	Severe oedema	Very large, necrotic, spreading	Not done
4-72	Acetic acid 5%	0.1	Very slight	Moderate 3.5 x 3 cm., spreading	1 x 1 cm.
4-73	Acetic acid 0.25%	0.1	Very slight	Moderate 3 x 2.5 cm., spreading	1 x 1.5 cm.
	Infection of Similar Areas in Immune Animals				
2-45	Acetic acid 2%	0.5	Moderate oedema	Large 5.5 x 5 cm. No spread	Moderate 4 x 4 cm.
2-68	Acetic acid 2%	0.5	Very slight	Small 2 x 2 cm. No spread	Small 1.5 x 1.5 cm.
2-78	Croton oil	0.1	Severe oedema, moderate necrosis	Large 7 x 3 cm. No spread	Small 1 x 1 cm.
2-81	Croton oil	0.1	Moderate oedema, slight necrosis	Large 8 x 3 cm. No spread	Small 1.5 x 1.5 cm.
2-99	Xylol	0.2	Moderate lesion red, localized	Moderate 4 x 4 cm. No spread	2 x 2 cm.
3-02	Xylol	0.2	Moderate lesion red, localized	4.5 x 4 cm. No spread	Small 1.5 x 1 cm.

ing a large necrotic lesion which usually proved fatal within a few days. Even when the amount of irritant was very small the dissemination of bacteria seemed to be increased. 24 hours later the infected irritated site was redder and larger than a control infection made elsewhere in normal skin. However, like a filtrate-treated area, no necrosis appeared at the areas receiving small amounts and healing

began much sooner. This confirms the work of Gay and others that the intensity of the previous injury often determines the outcome of local infection. (See Table III.)

Animals immunized to *B. lepisepticum* were treated similarly. Twenty-four hours after injecting an area with one of the above-mentioned irritants, the site was inoculated with *B. lepisepticum*. The infection spread throughout the inflamed area producing a lesion proportional in severity to that of the preliminary reaction. The immune animals never became ill, the lesions did not spread beyond the limits of the chemical irritation, necrosis was not so extensive, the discharge from the lesion was more purulent and, most striking of all, healing began early and developed more rapidly at the site previously inflamed than in a control infection in the same animal.

Immune serum injected mixed with the irritant is quite effective in controlling an infection induced the next day in the irritated area. A small series of normal rabbits received 0.4 cc. immune serum mixed with 0.1 cc. croton oil or 0.5 cc. of 2 per cent acetic acid, and 24 hours later, the lesions were inoculated with organisms but showed only slight infiltration which healed rapidly in the portion receiving the immune serum, but outside this protected area showed the characteristic necrotic spread. This observation is of some interest when contrasted with the fate of immune serum injected with a protein antigen to be described in the next experiments.

Infection of Skin Areas Reacting to a Coagulable Protein to Which the Animal Has Been Previously Sensitized

When organisms are introduced into a wheal of reacting skin of an animal sensitized to a coagulable protein, a decreased tissue resistance is observed similar to that obtained when tissues are inflamed by chemical irritants.

Normal animals were sensitized to egg albumin by injecting 1 cc. of egg solution subcutaneously at 5-day intervals for 15 days. When 0.2 cc. of egg solution was then injected intradermally the familiar pink, oedematous lesion of hypersensitiveness developed in 24 hours. Organisms injected into it spread with amazing rapidity throughout the whole reacting area, so that cultures made 18 hours later were strongly positive 10 cm. or further from the point of inoculation. Rapid necrosis appeared and the animals practically always succumbed. (See Fig. 1.)

The extent of the lesion in the first 24 hours was in proportion to the degree of hypersensitiveness shown by the animal.

We have studied the effect of immune serum on this type of infection and have obtained results which may throw some light on the nature of the underlying processes:

I. Infection of Egg-Reacting Sites in Animals Immune to the Infecting Organism

Rabbits were selected which had survived an infection with *B. lepisepticum* and which had protective substances demonstrable in the serum in such concentra-

TABLE IV

Results of Infecting Areas of Egg Reacting Skin in Animals Immune to B. lepisepticum and Sensitized to Egg Protein

Rabbit number	Intensity of skin reactivity to egg protein	Extent of infection in egg reacting site after 24 hours		Extent of infection in control site after 24 hours	
3-98	Marked oedema Sl. hemorrhagic	Large	8 x 4 cm.	Small	0.7 x 0.7 cm.
4-00	Marked oedema Mod. hemorrhage	Large	7 x 4 cm.	Small	1 x 0.7 cm.
2-72	Marked oedema	Moderate	6.5 x 5 cm.	Small	2 x 2 cm.
2-68	Moderate oedema Sl. hemorrhagic	Moderate	5 x 4.5 cm.	Small	1 x 1 cm.
2-66	Moderate oedema	Moderate	4.5 x 3.5 cm.	Small	3 x 2 cm.
3-87	Slight	Small	2.5 x 2 cm.	Small	1.5 x 1.5 cm.
3-88	Very slight	Small	1.3 x 1.2 cm.	Moderate	2.3 x 3 cm.
3-89	Very slight	Small	1.2 x 1.5 cm.	Small	1.5 x 1.3 cm.
3-99	Very slight	Small	1.7 x 1.7 cm.	Small	2 x 2 cm.

tion that 0.2 cc. injected into the skin of susceptible rabbits protected locally against a large number of *B. lepisepticum* injected into the same site.

These immune animals were sensitized to egg albumin as outlined above, skin-tested with egg, and the reacting skin inoculated with virulent *B. lepisepticum* 24 hours later. A control injection with the organism was made in the opposite flank.

In many animals which showed a strong reaction to egg protein, there appeared a large red lesion which spread to the margins of the reacting area but never beyond. The control sites were always small red papules or pustules which never attained a comparable size and healed rapidly. (See Table IV.)

The importance of this is obvious in explaining the development and persistence of local lesions in individuals having a high humoral im-

munity. On the one hand the cells locally may become so altered that the immune bodies of the blood do not penetrate into the lesion or, on the other hand, the cells are so injured that the immune bodies no longer exert the usual protecting mechanism. In animals which did not react strongly to the egg there was quite the opposite effect. The lesions were actually smaller than in the controls and healed more rapidly.

TABLE V

Infection by B. lepisepticum of Areas of Skin Reacting to Egg into Which Bacterial Immune Serum Was Injected Mixed with the Egg Antigen

Rabbit number	Amount of egg protein in skin	Amount of serum mixed with egg	Intensity of skin reactivity to egg serum mixture (after 24 hours)	Infection in egg reacting site (after 24 hours)		Control infection into normal skin of anima (after 24 hours)	
	cc.	*cc.*					
4-28	0.3	1	Marked	Mod.	6.5 x 5.5 cm.	Small	2.5 x 2.5 cm.
4-30	0.3	0.2	Mod. oedema	Mod.	4 x 3.5 cm.	Small	1 x 1 cm.
4-22	0.3	2	Marked oedema	Large necrotic	7 x 5 cm.	Small	2.5 x 1.5 cm.
4-33	0.3	0.2	Slight	Small	1 x 1 cm.	Small	1.5 x 1.5 cm.
4-25	0.3	0.4	Marked oedema	Large	9.5 x 5 cm.	Small	2.5 x 2.5 cm.
4-44	0.3	4	Marked	Large	8 x 5 cm.	Mod.	3 x 2.5 cm.
			Controls Using Normal Serum Mixed with Egg				
4-29	0.3	0.1	Marked	Large necrotic	7.5 x 5 cm.	Small	2.5 x 2 cm.
4-35	0.3	0.2	Slight	Small	2.5 x 2.5 cm.	Small	1 x 1 cm.
4-24	0.3	0.2	Moderate	Large	8 x 5.5 cm.	Small	2.5 x 2.5 cm.
4-26	0.3	0.4	Moderate	Large	6.5 x 5.5 cm.	Small	1.5 x 1 cm

II. Infection of Egg-Reacting Sites into Which Immune Serum Is Injected Simultaneously with the Egg Antigen

Normal rabbits sensitized to egg were given intradermally 0.3 cc. of egg solution mixed with varying amounts of immune serum of recognized potency. 24 hours later a large reacting area was found which was identical to that produced by egg alone, though perhaps somewhat larger and redder. When such an area was injected with *B. lepisepticum* the spread was just as rapid and necrosis just as extensive as in the animals receiving egg alone, or in the controls receiving normal serum mixed with the egg. (See Fig. 2 and Table V.)

From this experiment it is obvious that either, (1) the immune serum has diffused out of the lesion or, (2) has been denatured in some way or, (3) the cells have been so injured that they no longer profit by its presence. The latter hypothesis can be excluded by the next experiment.

III. Infection of Egg-Reacting Sites When Immune Serum and Organisms Are Injected Simultaneously

Normal rabbits were sensitized to egg as outlined above. Reacting areas were produced by injecting 0.3 cc. of egg intradermally. These wheals were injected with *B. lepisepticum* with which was mixed 0.1 or 0.2 cc. of immune serum. The local protection was striking. No lesion developed in the region of the injection, but when the dose of serum was small and the infecting dose of organisms large, the infection appeared at the periphery of the egg-reacting site where there were necrosis and other manifestations of infection. (See Fig. 3.)

It is evident that immune serum, if present, is a potent protective agent, even in the presence of injured cells. This serum seems more accessible to the injured cells of this type of lesion when injected into the tissues than when supplied through the general circulation. Normal serum has no effect when injected with the bacteria.

IV. Infection of Egg-Reacting Sites Which Are Induced by Injecting Bacterial Immune Serum with the Egg Antigen and in Which Normal Serum Is Injected with the Infecting Organisms

It seemed conceivable that the immune serum present during the antigen antibody reaction might be altered but not destroyed, and the following experiment has been devised to learn whether the immune serum could be reactivated by normal serum which itself has no protective value.

Normal rabbits were sensisized to egg and the usual hypersensitive reaction was induced by injecting intradermally 0.2 cc. of egg solution mixed with 0.5 cc. of immune serum on each side of the animal. 24 hours later the areas were injected with *B. lepisepticum* and, in addition, normal serum was injected into the reacting area, sometimes into the same site as the organisms, sometimes in another part of the wheal. The other side was infected with an equal dose of organisms but no normal serum was added. There was striking protection in the area receiving the normal serum, while the area receiving only bacteria showed the usual fulminating lesion.

TABLE VI

Wheals Induced in Both Flanks of Egg-Sensitized Rabbits by Injecting a Mixture of Egg and Bacterial Immune Serum. Infected 24 Hours Later, Injecting at Time of Infection Normal Serum in One Wheal, Heated Serum in Other

Number of animals	Amounts of egg and immune serum in mixture	Wheal receiving fresh normal rabbit serum at time of infection			Control wheal receiving only heated normal rabbit serum, or no serum at time of infection		
		Severity of preliminary wheal	Amount of normal serum injected	Result of infection	Severity of preliminary wheal	Substance injected with infection	Result of infection
			cc.				
4-48	0.3 cc. egg 0.4 cc. immune serum	Moderately severe	0.3	Small healing lesion, 2 x 2.5 cm.	Moderately severe	Nothing	Large spreading lesion, 8.5 x 6 cm. Necrosis at dependent part
4-57	0.3 cc. egg 0.6 cc. immune serum	Moderate reaction	0.4	Lesion moderate size, 7 x 3.5 cm. Healed promptly	Moderate reaction	Nothing	Large spreading necrotic lesion, 8.5 x 7 cm.
4-55	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.4	Moderate healing lesion, 8 x 4 cm.	Moderate reaction	Nothing	Large spreading necrotic lesion, 4 x 7 cm.
4-58	0.3 cc. egg 0.6 cc. immune serum	Moderate reaction	0.4	Small healing lesion, 3 x 3 cm.	Moderate reaction	Nothing	Large spreading necrotic lesion, 8.5 x 6.5 cm.
4-71	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.2	Moderate size, flat, sl. necrosis, healing, 5.5 x 5 cm.	Moderate reaction	0.2 cc. heated rabbit serum	Large spreading necrotic lesion, 9 x 6 cm.
4-74	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.4	Pale, healing, 4 x 4 cm.	Moderate reaction	0.4 cc. heated rabbit serum	Large spreading necrotic lesion, 9.5 x 5 cm.
4-67	0.2 cc. egg 0.4 cc. immune serum	Severe hemorrhagic	0.4 (Guinea-pig serum)	Small, pale, healing, 3.5 x 3.5 cm.	Moderate reaction	0.4 cc. heated guinea-pig serum	Large spreading necrotic lesion, 11.5 x 4 cm.

This experiment indicates the reactivation of the inert immune serum by normal serum. Experiments in a small series of animals indicate that the restoration of activity to the immune serum is due to the addition of *complement* or some other thermolabile substance.

Animals sensitized to egg were injected with 0.2 cc. of egg and 0.4 cc. of immune serum into the skin of both flanks. 24 hours later the wheals on each side were infected with equal doses of *B. lepisepticum*. At the same time 0.2 cc. to 0.4 cc. of fresh *normal* rabbit or guinea-pig serum was injected into one wheal while a similar amount of the same serum *heated* at 60° for 20 minutes was injected into the opposite wheal. Within 24 hours the wheal receiving the heated normal serum showed an extensive necrotic infection, while the wheal receiving the unheated normal serum showed definite evidence of healing. (See Table VI and Fig. 4.)

Further study is being made of the nature of this phenomenon and the indications are that some complement-like substance is used up in the egg-antibody reaction. Such a substance is probably necessary for the effective utilization by the cells of the immune serum which is present in the lesion but is inert.

DISCUSSION

It has been demonstrated in the preceding experiments that extensive tissue injury, due either to chemicals or to an intense antigen-antibody reaction allows pathogenic bacteria to disseminate with remarkable rapidity. This is observed even in animals with high humoral immunity though in such animals infection does not usually extend beyond the injured tissues. Such a mechanism of previous injury may be of importance in explaining the rapid development and persistence of large, local infections even in the presence of a high degree of humoral immunity.

Certain of the actions of immune serum in focal infection seem clear from these experiments. When preliminary injury is intense the tissues involved seem to become segregated from the body as a whole, so that circulating immune substances do not reach the cells of this portion in spite of the marked hyperaemia. In areas thus isolated pathogenic organisms may thrive and destroy the unprotected tissues. However, the cells outside, abundantly supplied with circulating antibodies, withstand the processes of infection as do the wandering cells

entering the lesion. Hence, in the immune animal the lesion fails to spread, exudate from it is more purulent in character and repair proceeds more efficiently. On the other hand when the local damage is slight, no such tissue segregation occurs. The supply of immune substances may be actually increased by the irritant. In such cases healing begins almost at once.

In the type of injury produced by a local antigen-antibody reaction, a segregation of the area is also striking. But an additional factor is introduced which lowers resistance, for bacterial immune serum present during the reaction fails to exert a protective influence unless unheated complement is added. This using up of a complementary substance perhaps demonstrates a mechanism by which specific immune processes may be disturbed in disease. In addition, one would not expect the administration of therapeutic serum intravenously to be effective against such a segregated lesion.

In the absence of specific immune substances, it is important to the host to restrict the invading organisms to a localized area. It has long been assumed that the allergic animal is better equipped with such a localizing mechanism. In Table I it is seen that strong reactors withstand infection better than weak reactors. If, however, a large lesion develops the animal quickly loses its skin reactivity and the infection spreads just as actively as in the weak reactor. Furthermore, the allergic reaction may be so severe and the local damage so great that the inflammatory zone becomes analagous to the wheal of an antigen-antibody reaction and the infection instead of being restrained spreads with great rapidity.

It must also be emphasized that allergy is not the only tissue factor to be considered in localizing infections. Our observations on *B. lepisepticum* quite confirm Rich's (10) findings with the tubercle bacillus that localization of the lesion often depends upon an intrinsic immunity of the cells against the injurious effects of infection. This may be easily demonstrated by the fact that a temporarily desensitized allergic animal without demonstrable humoral immunity may possess the capacity to restrain the spread of an infecting organism.

In spite of these variations in the influence of allergy on the course of infection our observations lead us to the belief that allergy is more than a "concomitant phenomenon" of the tissue immune state, and a

number of facts regarding the tissue reactions in an allergic animal can be cited which tend to confirm this view.

1. It has been shown that mild irritants such as broth filtrates when injected several hours previous to infection tend to protect locally even in the absence of general immunity. This preliminary inflammation is seldom comparable in extent and intensity, or in the amount of oedema observed in the wheals produced by egg in the sensitized animal, or in the lesion produced by chemical irritants.

2. Within a few minutes after the introduction of a mild irritant into the skin of a strong reactor the endothelial cells of the area become highly permeable on the *vessel lumen side* as is illustrated by injecting India ink intravenously during the first few minutes of the reaction and noting the phagocytosis of ink by the vascular endothelium. Negative reactors practically never show this phenomenon (11).

3. A few hours after injection into the skin of a bacterial filtrate to which the animal responds, the endothelial cells undergo a peculiar alteration so that if homologous filtrates or certain heterologous bacterial filtrates are injected intravenously, a hemorrhagic necrosis occurs at the site of skin injection. After an animal develops humoral immunity this phenomenon no longer takes place (12, 13). If, however, the homologous filtrate is injected into the site of the skin test no hemorrhagic necrosis occurs. On the contrary, an accelerated skin reaction develops in which redness and swelling appears within a few hours and begins fading many hours before a control test in a normal area of skin.

These observations make us believe that the endothelial cells of a strong reactor are exceedingly sensitive to products of certain bacteria and that within a relatively short time they become, on the tissue side, impervious to these products, perhaps through some protoplasmic alteration. Such stimulated cells are not readily destroyed by the products of infection present in the tissues but may be seriously changed if such products are absorbed and reach them through the medium of the vascular system which would be analogous to the hemorrhagic necrosis following the intravenous injection of filtrate. Clinically such lesions are not uncommon during the course of severe infections.

CONCLUSIONS

1. Animals showing natural bacterial allergy to filtrates of *B. lepisepticum* survive infection by this organism more frequently than weak reactors. This increased resistance is manifested by better localization of infection.

2. Bacterial filtrates injected into skin 24 hours before infection exert a non-specific protection of that area against the organism, even

in susceptible animals. The cells of this protected area seldom undergo necrosis when infected.

3. Severe injury of tissues either by chemicals or an antigen-antibody reaction produces a loss of local resistance even in immune animals. Mild injuries have the opposite effect. It is believed that in cases of severe injury, the affected areas undergo a segregation from the circulating antibodies.

4. When bacterial immune serum is injected with a protein antigen into the skin of a sensitized animal, a local alteration occurs in which substances necessary for the effective action of the immune serum are destroyed.

5. A protective action is restored to the altered immune serum by addition of complement to the lesion.

6. It is felt that allergy is not the chief mechanism in cellular resistance to infection, however data are advanced which suggest that allergy does exert local protection by acceleration of the immune processes and by rendering the cells locally refractory to further injury.

7. Chronic infection by a single strain of organism excites cellular reactivity to many strains of bacteria often unrelated biologically. Hence a non-specific mechanism for localizing infections throughout the body may be induced.

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EXPLANATION OF PLATES

PLATE 18

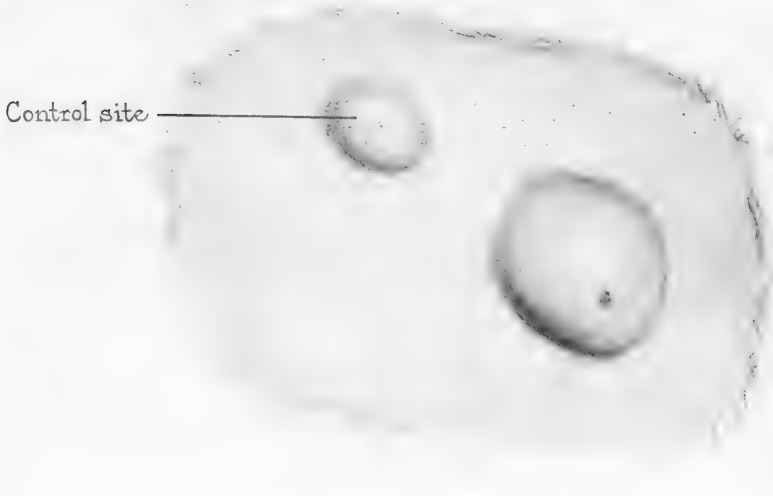
FIG. 1. No. 4–26. Animal sensitized to egg. Wheal induced with 0.3 cc. egg protein 24 hours before infection in it and in a control site of normal skin. Drawing, made 24 hours after infection, shows relative size of lesion.

FIG. 2. No. 4-25. Animal sensitized to egg. Wheal induced by injecting a mixture composed of 0.3 cc. of egg protein and 0.4 cc. *B. lepisepticum* immune serum. Infected 24 hours later. Drawing, made 24 hours after infection, shows huge infection in spite of immune serum.

PLATE 19

FIG. 3. No. 4-27. Wheal induced as in Fig. 1. *B. lepisepticum* immune serum 0.2 cc. injected into wheal at X with infecting bacteria. Drawing, made 24 hours after infection, shows striking protection of the portion of the wheal receiving immune serum.

FIG. 4. No. 4-74, May 24, 930. Wheals induced in both flanks by injecting 0.2 cc. egg and 0.04 cc. bacterial immune serum in sensitized animal. Infected 24 hours later with equal dose of bacteria, but in one site 0.4 cc. normal rabbit serum was injected with organisms; in the other an equal amount of rabbit serum heated 60° for 20 minutes.



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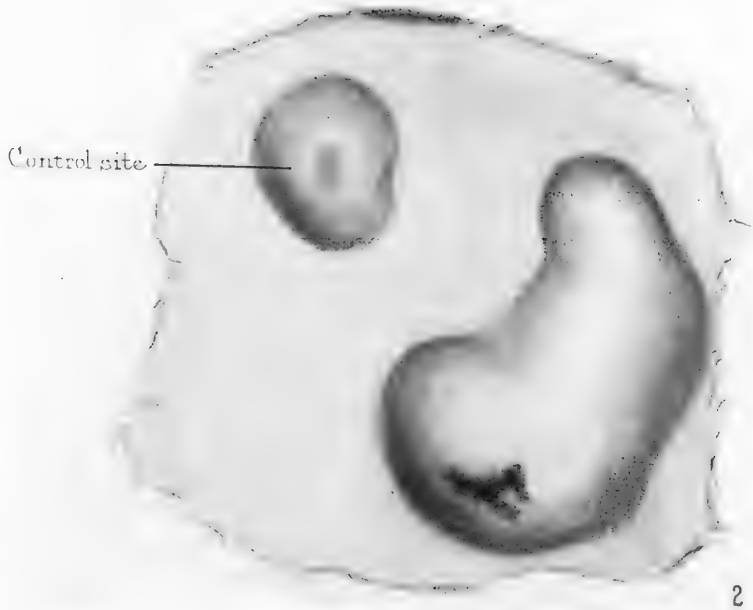

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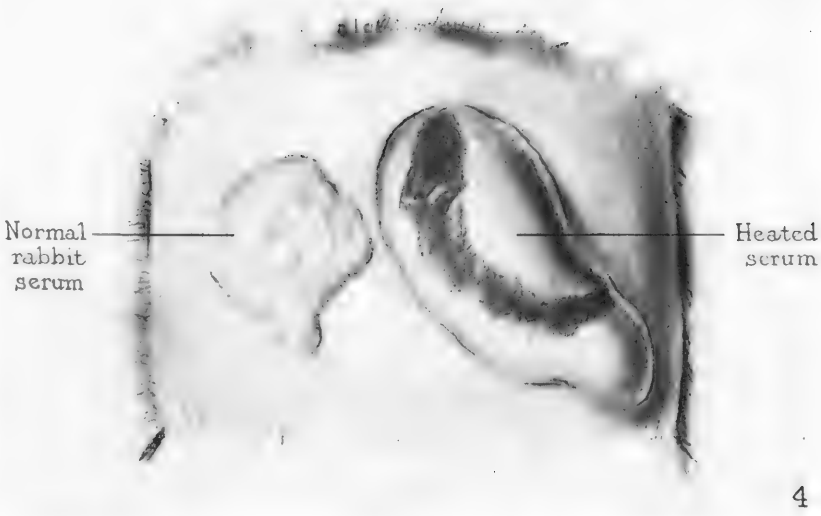

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HOST FACTORS IN THE PATHOGENESIS OF PNEUMOCOCCUS PNEUMONIA

I. The Reaction of the Lungs to Pneumococcus Autolysate in Sensitized Rabbits

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Plate 20

(Received for publication, June 16, 1930)

Allergy or hypersensitiveness to bacterial products is believed to be an important factor in modifying the reaction of the host to many infectious diseases. The localization of the antigen by a prompt inflammatory response in the allergic host has been considered by some as evidence that allergy is an expression of partial immunity, by others that it is one of increased susceptibility. To what extent this phenomenon may play a part in the pathogenesis and clinical course of pneumococcus pneumonia in man is unknown but has seemed worthy of study. The present investigation was consequently undertaken to observe the response of the lung to pneumococcus antigen in animals previously rendered hypersensitive to the pneumococcus. No attempt has been made to answer the question whether the allergic state is beneficial or harmful.

The existence of allergy in man to pneumococcus is suggested by the local reactions obtained in many human beings when various fractions or products of the pneumococcus are injected intradermally (1, 2, 3, 4, 5, 6, 7), and by the rapid development of the pathological lesion of pneumococcus pneumonia with its characteristic diffuse exudate. Lauche (8), in fact, largely on clinical and theoretical grounds, has strongly advocated the view that hypersensitiveness plays an important rôle in the pathogenesis of lobar pneumonia, though presenting no direct experimental observations in support of this conception.

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In contrast with man, young rabbits are rarely skin reactive to pneumococcus antigen and the intratracheal inoculation of virulent pneumococci in them usually produces a septicemia without pulmonary lesions resembling those found in human pneumonia. It seems logical, therefore, that the question has been raised whether the somewhat peculiar susceptibility of man to pulmonary infection by the pneumococcus and his capacity to localize the infection in the lung are related to allergy to that bacterium or its products and, if so, whether these characteristics are paralleled by cutaneous sensitivity.

Although experiments on the relationship of general allergy to pulmonary reaction have been reported by Schlecht and Swenker (9), who first sensitized guinea pigs to human serum by intraperitoneal inoculation and later caused the animals to inhale the same antigen with a resultant exudative inflammation of the lungs, comparable experiments with pneumococcus as an antigen have not been found in the literature. Wadsworth (10), however, has reported experiments that are highly suggestive. He immunized rabbits with bile and saline solutions of pneumococci and 2 weeks later was able to produce a diffuse inflammatory response resembling the pneumonia of man by injecting a virulent culture intratracheally. In repeating this work Kirkbride (11) assumed, but did not demonstrate the existence of sensitivity at the time of the intratracheal injection and, furthermore, she found the pathological picture confused by pulmonary infections caused by *B. lepisepticum* or *B. bronchisepticus*. She concluded "that the development of the diffuse exudative reaction in the lung is not due to an acquired hypersusceptibility but to intrinsic qualities possessed by the pneumococcus itself."

Experimental Procedure

Some of the conditions found in human beings can be simulated experimentally in animals by first making animals skin sensitive to pneumococcus antigen and then introducing the same antigen into the lungs of the sensitized animals. Inasmuch as the histological picture produced locally in a sensitized skin by the inoculation of antigen is accepted to be a true inflammatory process, one might expect the capillary bed of the lungs in the sensitized animal to respond as the capillary vessels of its skin, namely, by the outpouring of an exudate. In addition, if an inflammatory condition in the lungs could be produced by a sterile isotonic solution of pneumococci, harmless to normal non-sensitive animals, the result would appear to be of more significance than if living organisms were used, since many of the experimental difficulties associated with the use of whole organisms, whether living or dead, would be avoided. Consequently rabbits were sensitized to pneumococcus by several methods and their degree of

sensitivity determined at frequent intervals by intracutaneous injections of pneumococcus autolysates. At various intervals ranging from 15 to 62 days after the first sensitizing injection an intratracheal injection of autolysate was given to each rabbit. Twenty-four hours later the animal was killed and the lungs were examined for evidence of inflammation. Young white rabbits weighing between 700 and 1200 gm. were employed.

Preparation of Autolysates.—Saline autolysates were chosen because it has been shown (12) that they cause a more clear-cut reaction than a solution of pneumococci in bile or the whole bacteria, because the use of whole bacteria for lung injections might confuse the pathological picture by evoking a foreign body response, and because the errors of time and quantity attendant upon lysis *in vivo* could be eliminated.

Type II pneumococci of known virulence were used in preparing the autolysates. In most instances an avirulent strain which had been derived from a virulent Type II strain by growth in homologous immune serum was employed. This attenuated Type II pneumococcus was an intermediate between the true virulent S form and the avirulent R form, since it grew in small smooth colonies on blood agar but nevertheless showed agglutination reactions similar to those of rough pneumococci. In the concentrated autolysates prepared from it, the type specific soluble substance was demonstrated to be present by the precipitin reaction, but only in very small amount. In a few instances a highly virulent smooth strain was employed. The pneumococci were grown in buffered, 0.5 per cent dextrose, meat infusion broth for 6 to 7 hours at 37°C. The growth was then centrifuged and washed twice with saline. The bacterial sediment from 100 cc. of broth culture was suspended in 1 cc. of saline and was allowed to autolyze aerobically at 37°C. for 5 days. At the end of this period the tubes contained a milky material, which was sterile. Gram's strain showed only Gram negative amorphous débris. The pH of these solutions ranged from 5.4 to 5.8. Tenth normal sodium hydroxide was added to obtain a pH of 7.2 to 7.4. The whole mixture was centrifuged until the supernatant fluid became water clear, and the latter was then bottled without preservatives. Chemical tests on this solution showed a positive Biuret and a faint cloud with trichloracetic acid. The presence of pneumococcus protein and the type specific polysaccharide was demonstrated by the precipitin test (13).

With the autolysates made from either the avirulent or virulent Type II pneumococcus, none of the primary toxic skin reactions were observed. The hemotoxin described by Cole (14), and the necrosis producing factor described by Parker (15), had probably been eliminated by the 5 day period of autolysis at 37°C. under aerobic conditions. Nevertheless, in order to avoid any possibility of error arising from the presence of these toxic products, the autolysates were heated at 60°C. for 30 minutes, since it has been shown that the hemotoxin and the necrotizing

fraction are thermolabile. The purpura producing principle described by Julianelle and Reimann (16) may possibly have been present, but since no purpuric lesions were noted in the skin or lungs of non-sensitized rabbits and none in the lungs of sensitive rabbits there is no reason to suppose that, if present, it played any part in the results.

Standardization of Autolysates.—The autolysates were standardized on the basis of total nitrogen content, which varied from 150 mg. to 34 mg. per 100 cc. in different lots. Preliminary intradermal tests with an autolysate made from the avirulent pneumococcus and containing 125 mg. of nitrogen per 100 cc. gave a satisfactory range of intensity of skin responses from intradermal injections of 0.2 cc. (0.25 mg. N). Consequently that volume of each autolysate which contained 0.25 mg. of nitrogen was fixed as the unit skin test dose. It was found, however, that autolysates prepared from the virulent S strain of Type II pneumococcus and containing large amounts of soluble polysaccharide, required slightly less volume to elicit a reaction like the response from the standard injection unit than would have been expected on the basis of nitrogen content. It was inferred from this that some of the local inflammatory response might be caused by the specific polysaccharide. This factor in the case of autolysates prepared from virulent pneumococci was controlled by using a volume which was shown by actual inoculation tests to cause the same intensity of skin reaction as that caused by the standard dose of autolysate prepared from the avirulent organism.

Methods of Sensitization.—Various methods of sensitization were used and the animals are grouped accordingly. The details showing the number and spacing of injections for each rabbit are presented in Table I.

Group 1. Three rabbits (Nos. 6A, 7A, and 10A) received only intradermal injections of 0.2 cc. of avirulent Type II autolysate every third or fourth day for varying periods before intratracheal injection. These injections also served for the tests of cutaneous sensitivity.

Group 2. Five rabbits (Nos. 1B, 4B, 5B, 12B, and 14B) received intravenous injections of 0.2 cc. of avirulent Type II autolysate every 3 or 4 days for varying periods and in addition 0.2 cc. of the same autolysate intradermally for skin tests 24 hours before each intravenous injection.

Group 3. Four rabbits (Nos. 4C, 7C, 8C, and 11C) were treated with whole avirulent intermediate Type II pneumococci. Each received a single subcutaneous injecton of killed organisms, the dose in each instance being the growth from 50 cc. of a 6 hour 0.5 per cent dextrose infusion broth culture. In addition, Nos. 4C and 8C each received three intradermal skin-test injections of avirulent Type II autolysate, No. 7C six intradermal tests and No. 11C, five.

Group 4. Two rabbits received intravenous injection of avirulent Type II pneumococci. Rabbit 5C was given four injections, the first being of killed organisms, the other three of live organisms. The doses were the growth from 5 cc., 5 cc., 7 cc. and 7 cc., respectively of a 6 hour 0.5 per cent dextrose, infusion broth culture. Rabbit 4E received two injections of killed bacteria, each dose

being the growth from 10 cc. of broth culture. In addition No. 5C received seven intradermal tests with avirulent Type II autolysate, No. 4E, six.

Group 5. Four rabbits (Nos. 3F, 5F, 2G, and 6G) were sensitized by a single intravenous injection of killed virulent Type II pneumococci, the dose in each case being the growth from 5 cc. of broth culture. They were skin tested with autolysate at 3 to 5 day intervals over a period of 43 days in the case of No. 3F, 51 days in the case of No. 5F, 43 days in the case of No. 2G, and 54 days in the case of No. 6G.

Group 6. Two rabbits (Nos. 1K and 2K) were sensitized, each by a single intravenous injection of killed avirulent, rough, Type I pneumococci. The sensitizing dose consisted of the growth from 5 cc. of broth culture. Skin tests with avirulent Type II autolysate were done at 3 to 5 day intervals over a period of 45 days in each case.

Skin Tests.—The skin tests for sensitivity to pneumococcus were made by intradermal injection of the standard dose of autolysate in the skin of the flanks which had been shaved 48 hours previously. The days on which the skin tests were made and the degree of reaction exhibited at each test are shown for each rabbit in Table I. The reactions in sensitive animals appeared in 4 to 8 hours. Positive reactions consisted of local areas of erythema and edema which increased to a maximum size in 18 to 24 hours. Neither induration nor nodule formation nor the "secondary reaction" of Andrewes, Derick and Swift (17) were seen. Four degrees of intensity were recorded:

1. Slight (±). Erythema faint but definite. Edema very scanty or absent. Measurements taken in the short and long diameters showed extreme readings of 1 x 1 cm. and 6 x 2 cm.

2. Moderate (+). Erythema deeper. Definite edema present. Lesions on the average somewhat larger than in 1. Size varied between 1.5 x 2 cm. and 6 x 3 cm., the average being 2 x 3 cm.

3. Marked (±±). Erythema quite intense. Edema considerable. Average size about 3 x 4 cm.

4. Maximum (++). Very intense erythema, sometimes purpuric. Edema extensive with sagging skin. The lesions often required 72 hours to subside and superficial desquamation of the skin resulted frequently. No ulceration.

Although no gross necrosis with ulcer formation was noted, it is conceivable that the extreme reaction of tissue injury which has been reported by others (12) might have resulted from the use of a more concentrated autolysate. The skin test unit which was adopted for this study permitted a comparison of degrees of reactivity, and for that reason was considered most satisfactory.

Intratracheal Injections.—Twenty sensitized rabbits and seven non-sensitive normal controls were injected intratracheally with clear sterile isotonic pneumococcus Type II autolysates of standard nitrogen value. As shown in Table I, the injections were always made the day following a skin test in order to correlate the results as nearly as possible with the just determined skin sensitiveness of the

animal. The rabbits were prepared by shaving the neck and sterilizing the skin. A hypodermic needle was inserted between the rings of the tracheal cartilages and the autolysate was injected slowly while the animal was held with the fore part of the body slightly elevated. After completion of the injection the animal was kept with fore part elevated for 2 minutes. A standard volume of 5 cc. of saline was

Cutaneous Reactivity and Pulmonary Reactio

Group	Method of sensitization	Rabbit No.	Initial sk. test	Weeks 1 — Days 1	2	3	4	5	6	7	2	8	9	10	11	12	13	14	3	15	16	17	18	19	20	21	4	22	23	24	25	26
1	Avirulent pneumococcus Type II autolysate intradermally	6A	−				−					−				−					−				−				−		
		7A	−				−					+				+					±			−				−			
		10A	−				±			−					± ±			++	t	−											
2	Avirulent pneumococcus Type II autolysate intravenously	1B	−	s			±	s				+	s			−	s				−	s		−	s			−	s		−
		4B	±	s			± ±	s				±	s			±	s				−	s		±	s			−	s		−
		5B	−	s			±	s				−	s			±	s				± ±	s		± ±	t	+					
		12B	±	s			± ±	s				+	s			± ±	s				−	s		−	s			−	s		−
		14B	±	s			± ±	s				±	s			± ±	s				++	t	++								
3	Killed avirulent pneumococcus Type II subcutaneously	4C	±	s																						+					
		7C	−	s					−					±					±								±					
		8C	+	s																						−					
		11C	−	s					−					± ±					± ±								± ±	t	±			
4	Avirulent Type II pneumococci intravenously	5C	−	s			++	s				−	s			−	s			± ±						++					
		4E	+	s										s																	
5	Killed virulent Type II pneumococci intravenously	3F	−	s				+						± ±				−							± ±			+			
		5F	±	s			+					±				±					+					± ±			+		
		2G	+	s				+						± ±				−					−					±			
		6G	±	s				+						± ±				+					+					±			
6	Killed Type I-R pneumococci intravenously	1K	±	s			± ±					± ±				± ±					±				±				−		
		2K	+	s			++					± ±				++					±					+			+		

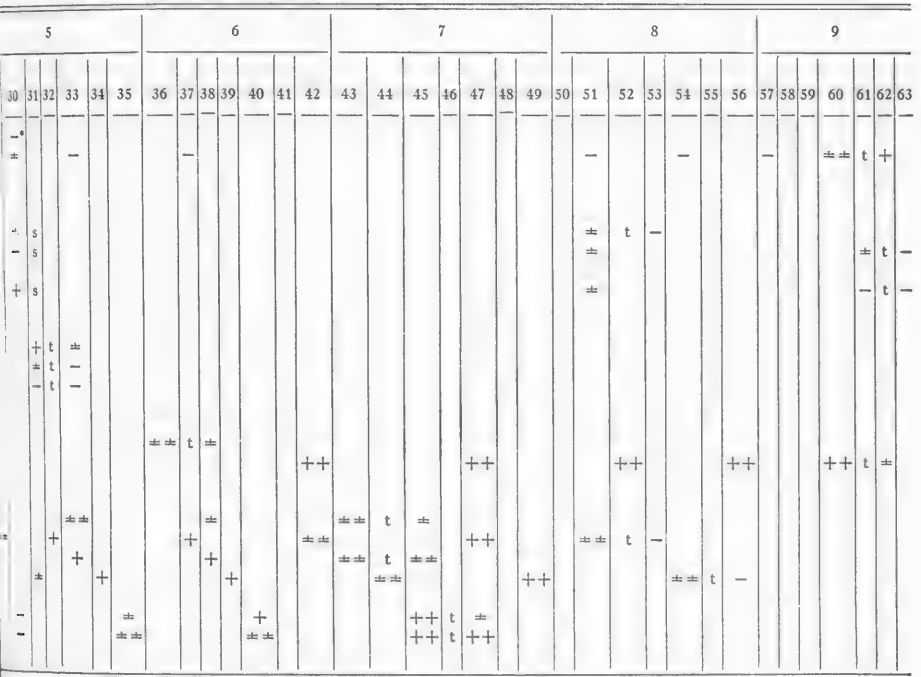
s = sensitizing injection; t = intratracheal injection of avirulent pneumococcus Type II autolysate.
* − to ++ signs following intratracheal injection indicate degree of acute exudative inflammation.
− = negative; ± = slight; + = moderate; ± ± = marked; ++ = maximum.

used and each dose contained 1 to 1.5 mg. of total nitrogen. Except for a variable amount of coughing, no symptoms were shown by the rabbits.

Examination of the Lungs.—The animals were killed 24 hours after intratracheal injection by inserting a needle through the cisterna magna into the base of the brain. This procedure was rapid and avoided the errors of technique which

etherization or air embolus entail. The chest was opened aseptically. One half of the right lung was excised and cultured in broth and on a blood agar plate. An injection of Zenker's formol solution was made *in situ* to preserve the contour and to prevent atelectasis. Histological sections were prepared and stained with hemotoxylin and eosin, Mallory's connective tissue stain, and MacCallum's

umococcus Autolysate in Sensitized Rabbits



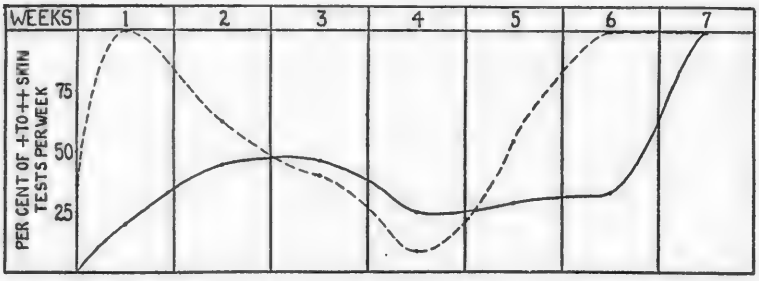
of skin tests and degree of cutaneous sensitivity are indicated by − to ++ signs.
gs.

modification of Goodpasture's bacterial stain. Grossly, no significant changes were observed on the surfaces, and sections of the fresh lung were not made. The cultures were sterile except in three cases. In two rabbits, Nos. 5F and 6G, whose lungs showed no acute inflammatory reaction, *B. bronchisepticus* was found; in a third rabbit, No. 3F, whose lung showed a pleural scar, *B. lepisepticum* was present,

but the pathological picture showed an acute exudate in the alveoli which was unlike the usual response to *B. lepisepticum*. Nevertheless, because of the presence of the bacteria, the results from this rabbit may be of doubtful significance.

Experimental Results

Cutaneous Sensitivity.—The course and degree of cutaneous sensitiveness to pneumococcus autolysate exhibited by each rabbit are shown in Table I. It will be noted in the first place that of the 20 rabbits, 9 showed no reaction on preliminary test, 7 showed a slight (±) reaction, and 4 showed a moderate (+) reaction; in the second place, that all but two rabbits (Nos. 6A and 8C*) exhibited moderate



TEXT-FIG. 1. The effect of existing hypersensitiveness on the course of cutaneous sensitivity to pneumococcus autolysate. Unbroken line, initially non-sensitive rabbits; broken line, initially sensitive rabbits.

to very marked cutaneous reactivity at some time subsequently during the course of the experiment; in the third place, that the course of the sensitiveness in general was wavelike in character with a markedly positive phase during the first 2 to 3 weeks, a negative or only slightly positive phase beginning in the third and running through the fourth into the fifth week with the return of a strongly positive phase in the sixth and seventh weeks; and lastly, that in spite of the general trend there was marked individual variation both in the degree and the course of sensitivity irrespective of the method of sensitization. Only

* 8C was not tested during the first 20 days and would probably have reacted during this period.

one obvious factor would appear to have exerted any significant effect on the course of events, namely, the initial state of sensitivity as determined by the preliminary skin test.

This is brought out in Text-fig. 1 in which it will be seen that the originally non-sensitive rabbits responded more slowly and less markedly during the first period of increased sensitivity than the originally sensitive rabbits. Subsequent to this, however, no significant differences between the two groups are apparent. Further discussion of the course and degree of cutaneous reaction in rabbits sensitized to the pneumococcus is not pertinent here and would obviously be of no significance in view of the marked individual variation exhibited and the small group of animals studied. It will consequently be reserved for a subsequent paper in which the results obtained in a larger series of rabbits will be presented.

Pulmonary Reaction.—An acute exudative inflammation of the lungs was found in a considerable number of the sensitized rabbits following the intratracheal injection of sterile pneumococcus autolysate (Table I). Histological examination showed that the exudate consisted largely of polymorphonuclear leukocytes and serum. Fibrin was not conspicuous and there was no necrosis nor hemorrhage. The extent of the inflammation varied considerably and may be divided into four degrees, as follows: (1) slight (±), denoting small focal areas of inflammation consisting of a polymorphonuclear leukocytic exudate lining some of the bronchioles and a thin layer of leukocytes and serum in the adjacent alveoli; (2) moderate (+), denoting a more extensive involvement with focal areas of exudate in the bronchioles and alveoli; (3) marked (± ±), denoting a still more extensive involvement with confluent areas of serous and cellular alveolar exudate; (4) maximum (++), denoting involvement of about one-quarter of the total lung with peribronchial, bronchiolar, and alveolar exudate of leukocytes and serum (Figs. 1 and 2).

The cultures of the lung made at autopsy were sterile in all but one (No. 3F) of the rabbits which showed an acute inflammation of the lungs, so that an existing infection would appear to be excluded as a cause of the acute lesion found in the sensitized animals. Furthermore, the pulmonary infections due to *B. bronchisepticus* or *B. lepisepticum* have been shown (18) to consist of a more destructive lesion

than that encountered here. Rarely an associated fibrotic lesion of the pleura was present. Obviously a lesion of this character could not

TABLE II

Relation of Pulmonary Reaction to Cutaneous Sensitivity

Group	Rabbit No.	Cutaneous sensitivity	Pulmonary reaction	Culture of lungs
		Skin test with pneumococcus autolysate at time of intratracheal injection	Acute exudative inflammation of lungs 24 hours after intratracheal injection of pneumococcus autolysate	
1	7 normal controls	−	−	No growth
	6A	−	−	No growth
2	12B	−	−	" "
	8C	−	−	" "
	1B	±	−	No growth
3	4B	±	−	" "
	7C	±	−	" "
4	4C	+	±	No growth
	5F	±±	−	*B. bronchisepticus*
	6G	±±	−	" "
	5C	±±	±	No growth
5	11C	±±	±	" "
	7A	±±	+	" "
	5B	±±	+	" "
	2G	±±	±±	" "
	10A	++	−	No growth
	4E†	++	±	" "
6	1K	++	±	" "
	14B	++	++	" "
	2K	++	++	" "

− = no reaction; ± = slight reaction; + = moderate reaction; ±± = marked reaction; ++ = maximum reaction.

† Much of the autolysate did not reach the lungs but was found at autopsy to have been injected into the peritracheal tissues.

be considered a part of the acute response. A careful examination of the lungs of many uninoculated rabbits often showed pleural and sub-

pleural interstitial thickening which was interpreted to be the result of a previous injury. This type of lesion was easily distinguishable from the acute exudative inflammation found in the sensitized rabbits. With Mallory's connective tissue stain these lesions showed strands of fibrous tissue, a feature which was never present in the acute lesions.

Relation of Pulmonary Reaction to Cutaneous Sensitivity.—The relationship between the cutaneous sensitivity of the rabbits and their pulmonary response to pneumococcus autolysate is shown in Table II in which the animals have been regrouped according to the degree of cutaneous reactivity exhibited at the time of the intratracheal injection.

Group 1 consists of 7 non-sensitized normal controls whose skin tests with pneumococcus autolysate were negative. None of these rabbits showed any pulmonary reaction following intratracheal injection of the autolysate.

Group 2 includes three rabbits which showed negative skin tests at the time of intratracheal injection. One of these, No. 12B, had previously been skin-sensitive but was in a negative phase at the time of the intratracheal injection. Another, No. 8C, was initially moderately skin sensitive but the only two subsequent tests made were negative. The third, No. 6A, had failed to become reactive at any time, although eight intradermal injections of autolysate had been given over a period of 28 days. None of these animals showed any pulmonary reactions.

Group 3 consists of three rabbits, Nos. 1B, 4B, and 7C, which showed slightly positive skin reactions at the time of intratracheal injection. None of these rabbits had become highly skin sensitive in response to the sensitizing injections and none had a detectable pulmonary reaction when killed 24 hours after intratracheal injection.

Group 4 includes but one rabbit, No. 4C. This animal showed moderate skin reactivity and a slight, but definite pulmonary response to the pneumococcus autolysate.

Group 5 includes seven rabbits which had marked cutaneous reactions at the time of intratracheal injection. All but two of these showed an acute exudative inflammation in the lungs, slight in two, moderate in two, and marked in one.

Group 6 contains five animals which showed extreme skin reactivity. Four of these showed acute inflammation in the lungs. No. 10A was negative; Nos. 4E and 1K showed slight but definite reactions; Nos. 14B and 2K showed the maximum degree of inflammation.

Rabbit 3F, although markedly skin sensitive and showing an acute exudation in the lungs has been excluded from the above tabulation because the culture yielded *B. lepisepticum.*

As may be seen from Table II, the results from intratracheal injection of pneumococcus autolysate in skin-sensitive rabbits in general parallel the degree of skin reactivity to the same antigen. In none of the 10 rabbits in which the skin was not reactive, whether non-sensitized controls or sensitized rabbits in a negative phase of cutaneous reactivity did the lungs show any pulmonary response when pneumococcus autolysate was brought into contact with the pulmonary tissues. Similarly, in none of the three slightly skin-sensitive rabbits did the lungs show any detectable inflammation. On the other hand 10 of the 13 animals showing moderate to extreme cutaneous sensitiveness showed clear-cut acute exudative inflammation in the lungs. The three negative results are not surprising in view of the mechanical factors of the experiment. Obviously, the technique of intratracheal injection lacked the fine degree of accuracy of the intradermal injections in which the dosage and the localization of the autolysate in a given skin area were quite definite. In the case of the intratracheal injections, there was no certainty that all the injected fluid reached the lungs and, granting that it did, other variables such as the greater surface area of the intra-alveolar spaces and the possible subsequent loss of antigen by coughing may have influenced the result. Because of these factors it is not unreasonable to believe that in some instances the antigen may not have been in contact with the pulmonary tissue for a sufficient period or that the concentration of antigen may have been too weak in any given area to be effective in causing an inflammatory reaction. From the above considerations, one could expect at best only an approximate parallelism between the cutaneous and pulmonary reactions.

DISCUSSION

The results of the experiments described above indicate that when a rabbit is rendered hypersensitive to the pneumococcus, the skin reaction to pneumococcus autolysate being taken as an index of the hypersensitivity of the animal, the pulmonary reaction to contact with the autolysate closely parallels the cutaneous response, being consistently negative in non-sensitive and only slightly sensitive animals, usually positive in the moderately to extremely sensitive ones. Although the parallelism so far as degree of reaction is concerned is not a strict one in

the sensitive group, a result hardly to be expected in a biological experiment of this type, the contrast between the non-sensitive and the sensitive groups is sufficiently striking to seem significant and to leave little doubt that the acute exudative inflammation of the lungs in the sensitive animals is dependent upon the allergic state of the animal rather than upon any inherently injurious substances in the autolysate.

By the use of whole pneumococcus autolysate devoid of the primary toxic principles so far described, the experiments have been circumscribed so that the results might give an uncomplicated picture of the pulmonary reaction which depends upon the allergic state alone. Whether the pulmonary reaction is due to allergy to pneumococcus nucleoprotein, to the specific carbohydrate, or to both, or even to other unidentified pneumococcus substances in the autolysate, is not elucidated, but presumably might be determined by the use of these substances in purified form.

The bearing of these experiments on the pathogenesis of pneumococcus pneumonia in man is, of course, problematical, in view of probable host differences and because live pneumococci, which possess properties not present in the autolysate, were not employed for intratracheal injection. The chief value of introducing active infection in the rabbits would have been to acquire data on the possible relationship of the allergic response to protection, but the conditions would have been so complicated that no assurance that the reaction was due to allergy would have resulted.

In spite of the difficulties inherent in the transfer of results obtained in experimental animals to conditions in man, it would nevertheless seem not improbable that the experiments reported above may have some significance in human pneumococcus pneumonia. The possibility that allergy plays an important rôle in the disease has long been surmised, and, as mentioned above, has been extensively discussed by Lauche (8) whose arguments have been summarized by Cole (19) as follows: "The facts cited by Lauche in support of this point of view are (1) that lobar pneumonia does not occur during the first 5 months of extra-uterine life; (2) that lobar pneumonia increases in frequency during the first decades until the maximum and constant figures are reached; and (3) that the occurrence of lobar pneumonia in the new-

born has only been observed if the mother is at the same time affected with pneumonia. According to this concept the new-born contract pneumonia because they have become passively sensitized from the mother. As a rule, infants do not acquire the disease during the first 5 months because they have not had an opportunity of being actively sensitized. With the widespread distribution of pneumococci, however, there are many opportunities for infection and consequent sensitization during the first decades. Lauche states that the allergic state is not a constant one. Like immunity, it is transient and varies in degree. In order to develop pneumonia the individual must be in a hypersensitive state. If this stage is passed the individual reacts normally to infection, for instance, with a coryza."

Experimental observations in man bearing on this problem consist almost entirely of studies on the cutaneous reactions of man to a variety of pneumococcus antigens, both during health and during the active and convalescent stages of pneumococcus pneumonia. A resumé of these observations (1, 2, 3, 4, 5, 6) shows, in general, that 50 to 85 per cent of normal persons have been found to react locally to an intracutaneous injection of pneumococcus antigen with the "delayed" type of skin reaction, that during the acute stage of pneumococcus pneumonia the skin is not reactive to these same antigens, and that during convalescence from the disease a large proportion of patients sooner or later develop cutaneous sensitiveness. In addition Tillett and Francis (7) have recently shown that the reactions of convalescents may be separated into two types—(a) an immediate wheal and erythema type of reaction in response to intradermal injections of the type-specific carbohydrate of the pneumococcus, first appearing at or about the time of crisis and (b) a delayed type of reaction in response to injections of pneumococcus nucleoprotein, usually appearing somewhat later in convalescence.

Any attempt to harmonize these observations in man with the theory set forth by Lauche must presuppose that the sensitive or allergic individual who contracts lobar pneumonia becomes rapidly desensitized, at least so far as the reactive capacity of his skin is concerned, during the stage of invasion and onset, presumably through the liberation of considerable amounts of pneumococcus antigen in the lungs and its absorption and distribution to the cutaneous tissues through the

circulation. Furthermore, if desensitization does occur, it would seem probable that it is a specific desensitization to the pneumococcus rather than a general non-specific loss of cutaneous reactivity since Poole, Bumstead and Blake (20) have found that patients acutely ill with lobar pneumonia, though failing to react to pneumococcus autolysates, nevertheless frequently give a positive "delayed" type of reaction in response to an intradermal injection of a soluble antigen prepared from *B. influenzae* similar to that exhibited by many normal individuals.

While the theory discussed above may seem plausible, it cannot be accepted as fact at present because an important link in the chain of evidence is lacking, namely, observations in man on his sensitivity to pneumococcus just prior to the onset of pneumonia. It is at this point that the experiments in rabbits here reported may have an important bearing on the problem, since a hypersensitive or allergic state in the rabbit was apparently a prerequisite for an acute inflammatory response of the lung to a pneumococcus autolysate, harmless in itself in the normal or non-sensitive animal. The analogy between the conditions in lobar pneumonia in man and those in the experimental animals is, however, far from perfect, and the actual state of man's sensitiveness to the pneumococcus prior to the onset of pneumonia must be determined before the problem can be further elucidated.

SUMMARY AND CONCLUSIONS

The present study was undertaken in order to determine whether animals exhibiting cutaneous hypersensitiveness to pneumococcus would show an acute inflammatory reaction in the lungs when pneumococcus autolysate was brought into contact with the pulmonary tissues and, if so, whether the pulmonary reaction might be shown to be due to the allergic state of the animal, rather than to intrinsic properties of the autolysate. Twenty young rabbits were sensitized to pneumococcus by various procedures and their degree of hypersensitiveness determined at frequent intervals over varying periods of time by means of intracutaneous injections of pneumococcus autolysate standardized on the basis of nitrogen content and so treated as to be devoid of the known toxic principles. Twenty-four hours after the last skin test each rabbit was injected intratracheally with the same pneumococcus autolysate. Seven non-sensitive controls were simi-

larly injected intratracheally. Twenty-four hours after intratracheal injection the rabbits were killed. The lungs were removed, a portion was cultured, and the rest was examined histologically. Of the twenty sensitized rabbits, three that showed no cutaneous sensitivity and three that were only slightly skin-sensitive at the time of intratracheal injection exhibited no detectable pulmonary reaction to the autolysate; eleven of fourteen that showed moderate to extreme cutaneous hypersensitiveness were found to have an acute exudative inflammation of the lungs. The exudate consisted largely of polymorphonuclear leukocytes and serum. It varied in extent from a slight focal exudate lining the bronchioles and adjacent alveoli to a very marked diffuse involvement of considerable portions of the lung. In all but one of the eleven the cultures of the lungs were sterile. The single animal showing a positive culture (*B. lepisepticum*) has been excluded. None of the seven non-sensitive controls showed any pulmonary reaction to the autolysate.

From these results it may be concluded that there is in rabbits a fairly close parallelism between cutaneous and pulmonary hypersensitiveness to pneumococcus autolysate and that the inflammatory response of the pulmonary tissue resulting from contact with the autolysate depends upon the allergic state of the animal rather than upon inherently injurious substances in the autolysate. The observations are in harmony with the theory that allergy may play a part in the pathogenesis of pneumococcus pneumonia in man.

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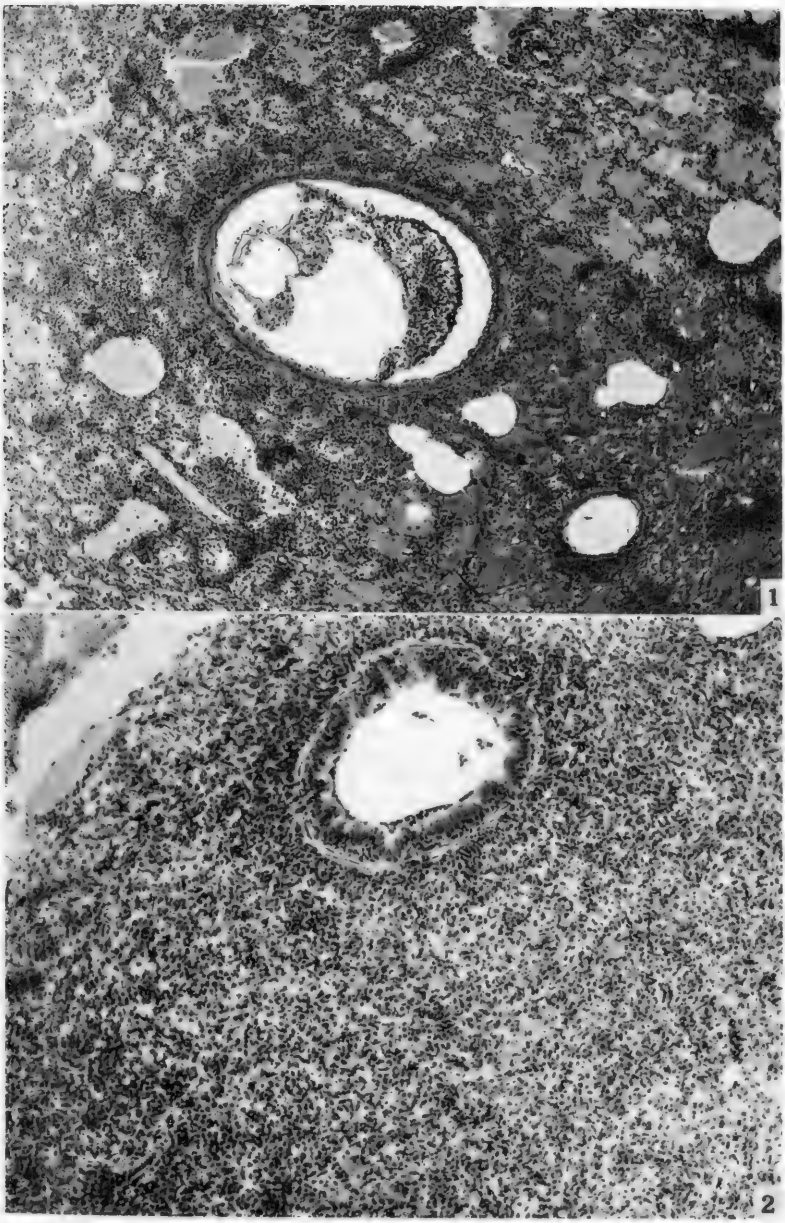
EXPLANATION OF PLATE 20

FIGS. 1 and 2. Rabbit 2K. Acute exudative inflammation of lungs 24 hours after intratracheal injection of pneumococcus autolysate.



(Sharp and Blake: Host factors in pneumococcus pneumonia. I)

UNIV. OF MICH.

STUDIES ON A-AVITAMINOSIS IN CHICKENS

I. Lesions of the Respiratory Tract and Their Relation to Some Infectious Diseases

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Plates 21 to 23

(Received for publication, June 19, 1930)

The diseased condition of chickens resulting from a lack of vitamin A in the diet is probably the most important of the nutritional diseases caused by A-avitaminosis in domestic animals. As pointed out by Beach (1) and Seifried and Schaaf (2), it causes serious losses on poultry farms. The clinical and anatomical picture closely resembles that found in some of the most important infectious diseases of fowls, chicken pox, coryza contagiosa, and infectious bronchitis. Golblatt and Benischek (3), Tyson and Smith (4), and Wolbach and Howe (5) have described the histological changes following a lack of vitamin A in the diet in guinea pigs, rats, and in one human individual. The characteristic changes are the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraocular glands and the genito-urinary tract. No studies have been made of the histological changes in A-avitaminosis in fowls. It is the purpose of this paper to report such studies and to compare the changes with those found in certain infectious diseases.

Material

The material used consisted of 16 cases of A-avitaminosis, of which 12 were produced experimentally and 4 were spontaneous. The diet employed for the experimental production of the disease was identical with that used by Beach and

* Professor extraordinary at the University of Giessen, Germany.

TABLE I

Showing Source and Kind of Material Used

Number of chicken		Food received	Age at beginning of experiment	First symptoms after	Dead after beginning of experiment	Gross lesions											Histological findings
						Eyes	Nasal fossa, cleft palate	Sinuses	Larynx	Trachea, bronchi	Cavity of the mouth, (excretory ducts of the glands)	Esophagus, crop	Proventriculus	Urates (kidney)	Urates (visceral)	Other lesions	
				days	*days*												
1	Experimental cases	Food mixture I alone	About 3 months		30 (Killed)	+	+++	+	+	+	−	−	−	−	−	−	Positive
2		"	"		40	70	++	+++	++	++	+++	++	+++	+	++	−	Oviductitis	"
3		"	"		45	76	−	++	++	++	+++	++	+++	−	+	−	−	"
4		"	"		48	79	−	++	++	+	++	+	++	−	++	+	−	"
5		"	"		44	85	++	+++	+++	+	++	++	+++	+	−	−	−	"
6		"	"		52	87	−	++	++	++	++	+	+	−	+	−	−	"
7		"	"		51	82	++	++	++	+	+++	+++	+++	−	−	−	−	"
8		"	"		58	80	++	++	+	−	++	+	+	−	−	−	−	"
9		"	"		54	75	±	+++	+++	++	+++	++	++	+	+	+	Oviductitis	"
10		"	"		59	77	+	+++	++	++	++	+	−	−	−	−	−	"
11		"	About 6 months		−	56 (Killed)	−	±	±	−	−	−	−	−	−	−	−	" *
12		"	"		−	81 (Died suddenly)	−	+	+	−	−	−	−	−	−	−	−	" *

13	Spontaneous cases	Lack of vitamin A	?	?	−	+	+++	+++	++	+++	−	−	−	−	−	Oviductitis	Positive
14		"	?	?	−	−	++	++	+	++	+	−	−	−	−	−	"
15		"	?	?	−	++	+	+	−	−	++	++	+	+	+	−	"
16		"	?	?	−	−	+	+	−	−	++	++	−	−	−	−	"
17–24	Controls	Food mixture I + cod liver oil + greens	6 about 3, 2 " 6 months old	−	−	−	−	−	−	−	−	−	−	−	−	−	Negative

* Main lesions in submaxillary and lingual glands.

by us in our earlier experiments. It consisted of 50 parts wheat bran, 50 parts wheat shorts, 50 parts ground barley, 10 parts meat scrap, 10 parts fish scrap, and 20 parts soybean meal. We used chickens about 3 months old, for according to our earlier experiments and in common with other investigators, we have observed that a much shorter period is required to produce the characteristic symptoms followed by death in young, partially grown animals, than in adults. This is brought out in the present experiments in which Chickens 11 and 12 (see Table I) that were about 3 months older than the other animals, developed symptoms much later and to a minor degree than did the younger ones. To insure the presence of vitamin D, all animals were exposed daily to sunlight or to ultraviolet light. The six controls (Nos. 17–24) received the same food mixture and in addition plenty of greens (alfalfa, cabbage) and 3 cc. of cod liver oil daily given with a dropper. All of the controls remained well, failing to show any gross pathological or histological lesions. No symptoms of rickets or of a deficiency of vitamin B were observed at any time during the experiment. Table I gives all details of the experiment together with the gross pathological findings.

Methods

Most of the tissues were fixed in 10 per cent formalin immediately after autopsy, but in a few cases Zenker's fluid was used. The tissues from the nasal passages and the sinuses were decalcified, some in 5 per cent nitric acid and some in 5 per cent formol-nitric acid. All tissues were imbedded in paraffin, and for special purposes frozen sections were made. For routine stains hematoxylin and eosin, van Gieson's stain, and Mallory's methylene blue phloxine, as modified by Davidoff (6) and McGregor (7), were used. In addition the following stains were used for special purposes: methylene blue, Gram's and Giemsa's stain,—the best results being obtained with the last, with tissues fixed in formalin as well as in Zenker's fluid, by the modification given by Wolbach,—Weigert's elastic tissue stain, Weigert's fibrin stain, fat stain with Sudan III and Scharlach R, oxydase reaction, the stains with methylgreen-pyronin and fuchsin-light green, with mucicarmine (Mayer), thionin, toluidin, cresylecht-violette, and Pasini's method with waterblue-orcein, which gave distinct results in staining the keratohyalin globules, and finally stains with ferric sulfur, calcium permanganate, Congo red (keratinization), and Hammerschmidt's stain for Guarnieri's inclusion bodies.

Gross Pathology

Nasal Passages and Sinuses.—Sagittal and gross sections through the nasal cavities and the communicating sinuses show all stages of a disturbance of the lining of the nostrils and cleft palate.

Early in the disease the turbinate bones (concha superior and inferior) are filled with seromucoid, water-clear masses, which by the application of slight pressure

can be forced out of the nostrils and cleft palate. Owing to the consistency of the exudate and the complicated structure of the nasal fossae, the vestibule becomes plugged and the exudate overflows into the paranasal sinuses, often early in the disease. On the other hand, the exudate may be forced through the cleft palate. filling up the region around the vomer with seromucoid masses. Later this exudate becomes transformed into white or slightly yellow caseous masses which are found between the turbinate bones, in the nasolacrimal duct, nasal vestibule, and sinuses, causing more or less swelling of the face (Figs. 1 and 2). This involvement of the nasal passages and the sinuses may be uni- or bilateral. If on one side, the turbinate bones and the sinuses may become so enlarged that they extend over the median line and compress the opposite nasal fossa. The characteristic eye lesions in this disease, which will be described in another paper with M. Westhues, are complicated by the blocking of the nasolacrimal duct. When the sinuses are completely filled, the bulbus of the eye, which cannot move in a ventral direction, is pressed against the os frontale and sometimes forced out in a lateral direction. The protrusion of the palate into the cavity of the mouth, often observed in virus-diphtheria, is not usually seen in A-avitaminosis. After the inflammatory products have been removed, the mucous membranes throughout the nasal passages and sinuses show a peculiar, thin, rough and dry membrane, which may also be found in the mouth and throat. Unattached masses of white caseous material are often present in the cleft palate and in the surrounding mucous membrane of the roof of the mouth.

Larynx and Trachea.—Almost regularly in A-avitaminosis of chickens marked lesions occur also in the larynx, trachea and bronchi. As shown in the table, they are found in the early as well as in the later stages of the disease.

Chickens 11 and 12 on a diet lacking vitamin A showed no gross lesions, but microscopically early changes were found in the glands of the mouth, esophagus and trachea. Those in the trachea were apparently the most marked and were probably the oldest. The lesions in the trachea have as yet received no attention, but we feel that they were of special importance as it is so difficult to distinguish them from those commonly found in roup, diphtheria, and infectious tracheitis. In addition to the lesions near the entrance of the pharynx, consisting of pustule-like patches which are due to collections of white caseous material in the mucous glands, glandula cricoarytenoidea, the pathologico-anatomical picture is characterized by a peculiar laryngitis, tracheitis, and bronchitis. The mucous membrane of the anterior end of the larynx on its ventral side and in the pointed angle which is formed by the cartilages of the larynx, often shows caseous crumbly white masses. Similar lesions are frequently found throughout the entire length of the trachea and often in the bronchi. In very early stages, however, they are much less marked and may be difficult to see. The mucous membrane is then covered with a fine

film or haze; it is dry, dull, and slightly uneven on the surface, whereas the normal membrane is moist. In other cases small nodule-like particles may be visible in or beneath the mucous membrane, especially in the upper part of the trachea. In later stages the gross lesions are much more striking and may easily be seen with the naked eye. More or less thin membranes cover the mucous membrane throughout the entire length of the trachea and the bronchi. In most cases these membranes are pulled off the underlying mucosa, thus forming a thin-walled continuous tube within the trachea and bronchi (Fig. 3), a condition that might be mistaken for infectious tracheitis. The smaller bronchi very often become completely plugged with these membranes causing bronchiectasis. In some cases the larynx shows the most marked changes while in others the trachea is more involved.

Histopathology

Larynx, Trachea, and Bronchi.—The changes in these structures are the more easily understood as the normal anatomy is less complicated than in the nasal fossae and adjoining sinuses.

The first lesion is in the columnar ciliated epithelium and is characterized by an atrophy of the cytoplasm and a loss of the cilia. Along with this protoplasmic change, the nuclei often present more or less marked karyorrhexis. The atrophying and degenerating ciliated cells hang like tufts on the basement membrane and later are pushed off and may form a pseudomembrane.

While this process is going on, there appear as islands beneath the original epithelium new cylindrical or polygonal cells either singly or in layers. These are especially marked in the trachea where they may appear as focal syncytial masses (Fig. 4). The cells evidently have their origin in the original columnar epithelium. They differ from the original cells in that their nuclei stain more deeply with basic dyes, and the presence of numerous mitoses indicates that they are dividing rapidly. Mitotic figures are especially numerous in the deeper layers of this newly formed epithelium. As these new cells become more numerous and form layers beneath or in place of the original epithelium, the cells nearer the surface are more and more flattened, the nuclei become larger and contain less chromatin. Later the superficial cells are quite flat, the cytoplasm is homogeneous and the nuclei are lost (Figs. 5 and 6). In this stage the cell boundaries become less clearly defined and at the surface the cells are desquamated as hard, dry, flattened scales. Pasini's waterblue-orcein method shows keratohyalin granules scattered irregularly in the cytoplasm of these cells. In the outer layers these granules sometimes fuse and form hyalin masses of eleidin. The process resembles very closely the incomplete keratinization of the mucous membranes, especially that of the esophagus and certain parts of the tongue of chickens. The columnar ciliated epithelium lining the trachea and bronchi and the epithelium of the submucous glands thus become transformed into squamous stratified keratinizing epithelium with typical intercellular bridges. Owing to the fact that the keratinized epithelium extends as a

continuous layer into the glands of the submucosa, the picture strikingly resembles that of the mucous membrane of the cavity of the mouth, and to a less extent of the normal skin. This is brought out in Fig. 6. Eosinophilic leucocytes which are normally found in the keratinized epithelium of the tongue and esophagus appear either scattered or in small foci. Other cells which may be associated with secondary bacterial infections also appear.

Along with the formation of this keratinized epithelium the original columnar cells are thrown off either singly or in layers. Some of these cells may appear practically normal, but the majority lose their cilia, the protoplasm takes the routine stains less intensely, and the cells later become distended so that they have the appearance of a ball or a balloon (Fig. 7). The chromatin in the nuclei of these cells becomes irregular and fragmented, and later only a few chromatin knots remain scattered irregularly in the balloon-shaped cytoplasm. Finally the cells appear as more or less homogeneous shadows, the whole process being that described by Unna as "balloon degeneration." This balloon degeneration involves not only the original epithelium but also the squamous keratinizing cells. Occasionally these degenerating cells may fuse and form what may be called "balloon giant cells." The cell boundaries may still be visible in these unusually large giant cells.

In the deeper layers of the new epithelium a reticular degeneration of cells may be found. The cells are enlarged, the cytoplasm is occupied by round vacuoles, sometimes to such an extent that it is only visible as a reticular network, the nuclei show numerous chromatin clumps, and nuclear material may be extruded into the cytoplasm. This type of degeneration is not as common as the balloon type.

The degenerating cells become separated and collect in the lumen of the trachea and bronchi in the form of a membrane in which numerous bacterial colonies appear. These bacteria may invade the cells and in cases become so numerous that the cytoplasm is filled with them and the nuclei sometimes destroyed (Fig. 7).

Stains with Sudan III and Scharlach R of the trachea at various stages of the degeneration show practically no fat. In a few cases a small amount of fat was present but this type of degeneration is far from common.

As the keratinization of the epithelial cells of the glands in the trachea progresses, the presence of mucus, as shown by special stain, becomes progressively less. In the early stages one may find keratinized epithelium in one portion of the gland and secreting mucus cells in another. Later, as the keratinized epithelium replaces the true glandular epithelium, mucin is found, if at all, in very small granules in the center of what remains of the original epithelium. When the glands become completely filled with keratinized squamous epithelium mucin is absent.

Associated with the loss of cilia and the decreased or absent secretion of mucin one would expect an invasion of the mucosa with bacteria. Organisms are found not only on the surface of the epithelium and between the epithelial cells but also in the glands of the tunica propria, in the tunica propria itself, and even in the submucosa. The degenerating cells of the epithelium soon become invaded by

bacteria and leucocytes may make their appearance. As the disease progresses bacteria and signs of inflammation become less marked. However, signs of bacterial infection are not always present, and in the fowl we feel that the keratinization of the epithelium is not related to the bacterial infection. In the rat, Tyson and Smith (5) came to the opposite conclusion.

In nearly all cases, a more or less marked cell infiltration in the tunica propria, partly around the numerous vessels, partly in a more diffuse distribution, is found (Figs. 5 and 6). According to the results of control investigations, the occurrence of lymphoid tissue in the tunica propria of the trachea is a normal finding, but in cases of A-avitaminosis its collection is so massive that there is no doubt of its pathological significance. The greater part of these infiltration cells are lymphocytes, to which are added typical histiocytes and a few plasma cells. In most cases some eosinophile leucocytes, red blood corpuscles and occasionally neutrophile leucocytes have also been found. In some early stages with extreme degeneration and proliferation of the original epithelium and a distinct edematous condition of the underlying propria, the occurrence of peculiar large cells, a type of histogenous mast cells, has been observed, especially in the upper layers. They are elongated or oval with a longish-oval nucleus which is rich in chromatin and sometimes shows a typical wheel form. Using Giemsa's stain, very small blue or blue-violet granules can be found in their cytoplasm. In general one has the impression that the upper layers of the tunica propria, situated directly beneath the epithelium of the surface, and the neighborhood of the glands are occupied more densely with these infiltrating cells than its deeper parts. Sometimes the reverse is true; in fact, the degree of the infiltration varies greatly in different cases. Mitoses are very seldom recognizable in these cells. Moreover, lesions may also be found in the endothelial cells of the infiltrated vessels, the lumina of which are frequently filled with lymphocytes. There is more or less swelling of these cells and a change in the chromatin structure of their nuclei. In some preparations the elastic fibers seem to be thickened and more separated. Usually these lesions are associated with edema. In the remaining parts of the trachea no particular changes could be discovered.

The same complex of lesions described in the trachea is also present in the bronchi and bronchioli. They are sometimes filled with desquamated keratinized epithelium, so that bronchiectasis is the natural sequence.

Nasal Cavities and Communicating Sinuses.—The lesions of the nasal passages and their sinuses have been studied on cross sections cut perpendicular to the roof of the mouth at several points between the nostrils and the posterior limit of the nasal cavity or bulb of the eye. The evolution, course, and final stage of the process are exactly the same as previously described in the trachea (Figs. 8, 9, and 10). It may be pointed out that in general all parts of the nasal cavities are

involved and that only some chronological differences in the involvement of the several regions can be observed. In the gland-free part of the nasal vestibule, regio vestibularis, lesions have been determined in all cases, not so much in the form of a real keratinization but rather of an increased proliferation of the superficial epithelial cells.

Typical and distinct lesions, as described in the trachea, are found in the respiratory mucous membrane of the roof of the nasal vestibule and especially in the median and dorsal area of the part known as the concha of the vestibule. One obtains the impression that the entire process in this and other parts of the nasal cavities (olfactory portion, respiratory portion) (Figs. 8, 9, and 10) begins in the mucous membrane epithelium and that the epithelium of the glands becomes involved a little later.

In addition to numerous keratohyalin corpuscles in the cells of the superficial layers of the newly formed squamous stratified epithelium and the appearance of eleidin (Pasini's method), the following lesions in the nuclei of the keratinizing cells have been encountered in the nasal cavities:—As a rule it has been observed, even in the deeper layers, that marked alterations of the nuclei are coexistent with the formation and appearance of keratohyalin granules in the cytoplasm. There is especially a marked fragmentation and extrusion of nuclear material (nucleoli) and furthermore a relative increase in the volume of the cytoplasm as compared with nucleus (Fig. 11). According to the investigations of Ludford (8), the same lesions may take place in the skin under normal conditions, while in a hypertrophied epidermis, under pathological conditions, these nucleolar changes are still more marked. It seems very probable, therefore, that the nuclear substances and particularly the nucleoli are connected in some way with the process of keratinization and the formation of keratohyalin granules. In addition the nucleoli after extrusion from the nuclei not infrequently change their staining properties. When stained by Hammerschmidt's method they resemble Guarnieri's inclusion bodies. Moreover, the nuclei of these cells from which the nucleoli have been extruded have the characteristic shape of nuclei in cells containing Guarnieri's bodies. It is worthy of note that in the pathological keratinization associated with chicken-pox Eberbeck (9) found the same type of lesion.

The sinuses communicating with the nasal cavity show essentially the same lesions as in the epithelium of the nose and trachea. These lesions are modified depending upon the anatomical structure of the cavity itself. In the paranasal sinuses the mucous membrane is thinner and contains only a few glands on the median wall, contrary to the opinon of Bittner (10). Keratinization occurs in the nasolacrimal duct and in the excretory duct of the lateral nasal gland, glandula lateralis nasi. The lateral nasal gland itself is only slightly involved,

the main lesion being in the ducts and collecting space while the acini themselves appear normal.

Bacterial invasion of the degenerating epithelium occurs in the nose just as it does in the trachea (Fig. 12). It is, however, more marked and the nasal cavity and fossa may become filled with degenerated cells and purulent exudate. The tunica propria becomes involved and in addition to the infiltrating cells one may find a slight edema and a swelling of the endothelial cells of the blood vessels.

Relation of A-Avitaminosis to Some Infectious Diseases of the Upper Respiratory Tract

The great variability of the clinical symptoms and pathological lesions in A-avitaminosis of the respiratory tract may make it difficult to differentiate this condition from coryza contagiosa, virus-diphtheria, and infectious tracheo-bronchitis. In the presence of a simple nasal discharge and a discharge from the cleft palate, with involvement of the sinuses, and in the absence of typical pustule-like lesions in the mouth and esophagus, there is a close resemblance to coryza contagiosa as well as to a certain stage of virus-diphtheria (Riedmüller (11)). Recent observations have shown that in such cases, from the clinical, gross anatomical, and even etiological aspect, a differentiation is quite impossible. Furthermore in later stages, after the sinuses and the eyes have become involved and when lesions are present in the cavity of the mouth (12), the A-avitaminosis frequently is almost indistinguishable from the commonly occurring roup and virus-diphtheria. The white color of the caseous masses associated with A-avitaminosis is usually considered to be valuable for the differentiation from the more yellowish products in roup and virus-diphtheria; yet these differences, according to our experience, are by no means distinct enough. Finally, in the absence of typical lesions in other organs, difficulties may arise in distinguishing the disease from infectious tracheo-bronchitis which in certain stages shows a very similar picture in the larynx, trachea, and bronchi.

It will be the object of further investigations to discover possible relations between A-avitaminosis and the development of these infectious diseases of the upper respiratory tract. In this problem, as well as for the purposes of differential diagnosis, the most important matter

is a positive recognition of a present lack of vitamin A. The histological lesions which occur in the respiratory tract and throughout the body even in early conditions, taken in connection with the more recent investigations of Wolbach and Howe (5), Goldblatt and Benischek (3), and Tyson and Smith (4), who were able to produce similar lesions in rats and guinea pigs by feeding a diet lacking vitamin A, seem definitely to prove the specificity of these lesions. It may be added that in cases of so-called "coryza contagiosa" without a lack of vitamin A quite different lesions have been found.

Only by means of the histological examination is it possible to differentiate the A-avitaminosis from the above mentioned infectious diseases of the respiratory tract.

SUMMARY AND CONCLUSIONS

1. The principal tissue changes in the respiratory tract of chickens caused by a vitamin A deficiency in the food are, first, an atrophy and degeneration of the lining mucous membrane epithelium as well as of the epithelium of the mucous membrane glands. This process is followed or accompanied by a replacement or substitution of the degenerating original epithelium of these parts by a squamous stratified keratinizing epithelium. This newly formed epithelium develops from the primitive columnar epithelium and divides and grows very rapidly. The process appears to be one of substitution rather than a metaplasia, and resembles the normal keratinization of the skin or even more closely the incomplete keratinization of the mucous membranes (*e.g.*, the esophagus or certain parts of the tongue of chickens). In this connection findings have been described which not only afford an interesting insight into the complicated mechanism of keratinization, but also show probable relations between keratinization and the development of Guarnieri's inclusion bodies. Balloon and reticular degeneration of the upper layers of the new stratified epithelium has been frequently observed. All parts of the respiratory tract are about equally involved in the process; and the olfactory region as well, so that the sense of smell may be lost. The lesions, which first take place on the surface epithelium and then in the glands, show only minor differences.

2. The protective mechanism inherent in the mucous membranes of

the entire respiratory tract is seriously damaged or even entirely destroyed by the degeneration of the ciliated cells at the surface and the lack of secretion with bactericidal properties. Secondary infections are frequently found, and nasal discharge and various kinds of inflammatory processes are common, including purulent ones, especially in the upper respiratory tract, communicating sinuses, eyes and trachea. The development of the characteristic histological process is not dependent upon the presence of these infections, since it also takes place in the absence of infection.

3. The specific histological lesions make it possible to differentiate between A-avitaminosis and some infectious diseases of the respiratory tract.

These studies we hope will serve as a basis for further investigations on the relationship between A-avitaminosis and infection in general.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Sagittal section through the head of Chicken 7 with A-avitaminosis. Masses of purulent exudate in the nasal passages. (Dead after 82 days on experimental diet.)

FIG. 2. Cross section through the head of Chicken 13 with A-avitaminosis (spontaneous case). White caseous masses between left concha inferior and nasal septum and in the corresponding sinuses.

FIG. 3. Trachea of Chicken 6 with A-avitaminosis. Thin membranes, partly in the form of a tube, consisting of desquamated epithelium. (Dead after 87 days on experimental diet.)

FIG. 4. Trachea, cross section (84.5 ×). Chicken 1. Beginning stage. Degeneration of the surface epithelium and separation from its underlying support. On the left side formation of a new stratified epithelium in a small focus. (Killed after 30 days on experimental diet.)

FIG. 5. Trachea, cross section (50 ×). Chicken 8. Complete replacement of the epithelium of the respiratory mucosa and its glands by a stratified keratinizing epithelium. Infiltration of the submucosa. (Dead after 80 days on experimental diet.)

PLATE 22

FIG. 6. Same as Fig. 5 (80 ×).

FIG. 7. Cross section through trachea (990 ×). Chicken 1. Newly formed stratified epithelium. At the surface several cells showing balloon degeneration. Foci of bacteria between the degenerating cells. (Dead after 30 days on experimental diet.)

FIG. 8. Nasal septum (50 ×). Chicken 6. Complete replacement of the surface epithelium by stratified keratinized epithelium. Desquamation of the superficial layers. The glands show an earlier stage of the process, atrophy of the original epithelium. Slight infiltration of the submucosa. (Dead after 87 days on experimental diet.)

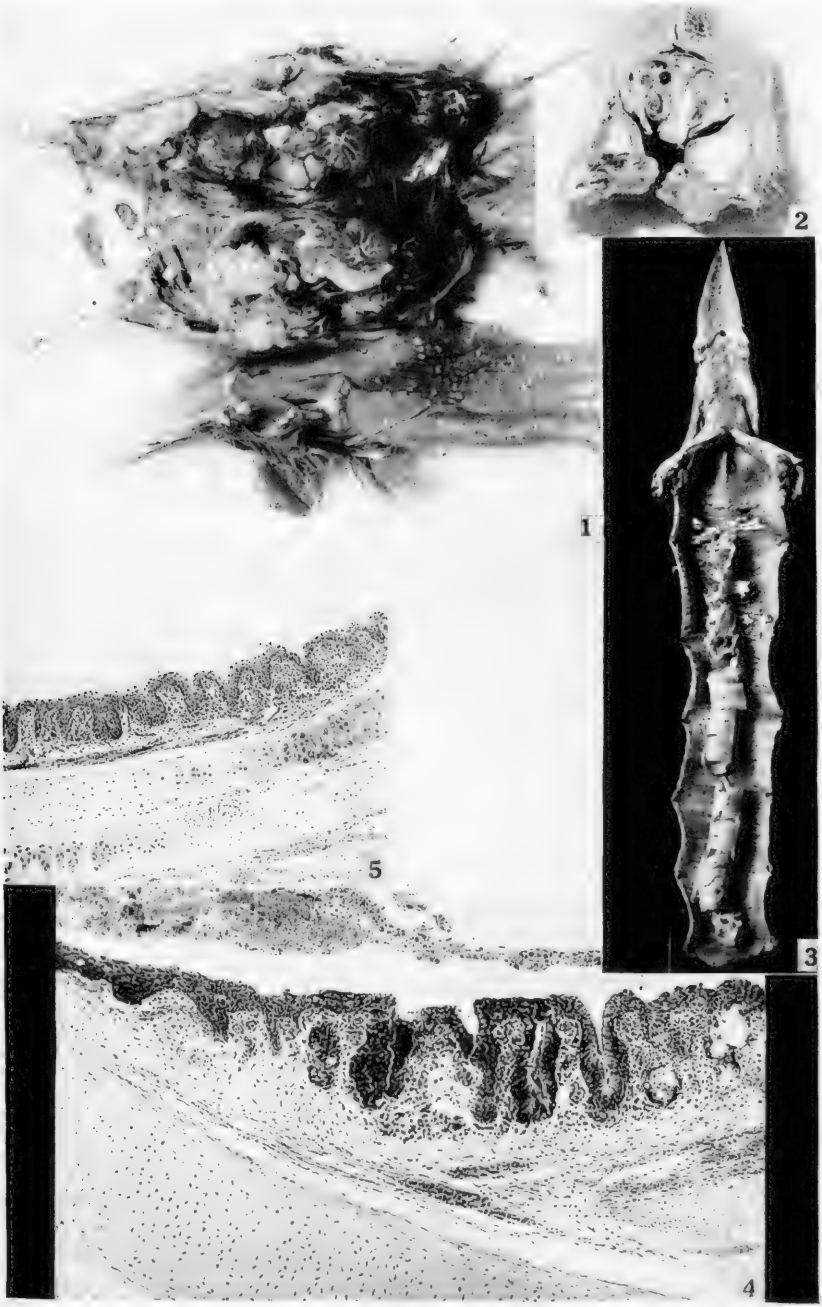
FIG. 9. Olfactory region (85 ×). Chicken 6. Advanced keratinization and desquamation of the replacing epithelium at the surface of the mucous membrane. The epithelium of Bowman's glands shows only the typical atrophy. Masses of exudate and desquamated epithelium. (Dead after 87 days on experimental diet.)

PLATE 23

FIG. 10. Respiratory and olfactory mucous membrane of the nasal fossa (55 ×). Chicken 5. Complete replacement by stratified keratinizing epithelium. The glands show an earlier stage of the process. (Dead after 85 days on experimental diet.)

FIG. 11. Gland of the nasal mucous membrane (1140 ×). Chicken 5. Showing numerous bacteria in an early stage of the process. (Dead after 85 days on experimental diet.)

FIG. 12. Nasal passages. Chicken 6. Cells of the superficial layers of the newly formed keratinizing epithelium, showing the extrusion of nuclear material into the cytoplasm (a), granules of keratohyalin (b), masses of eleidin (c), and intercellular bridges (d).



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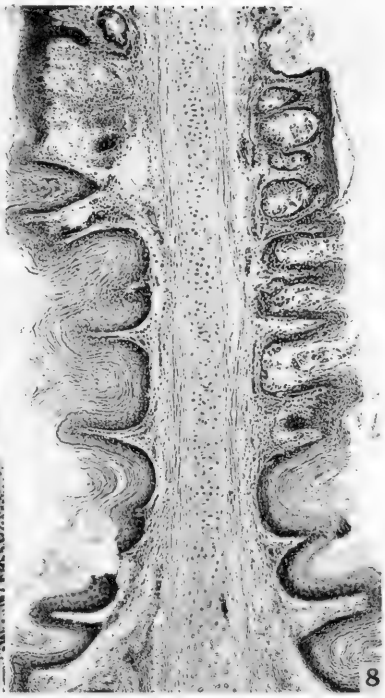

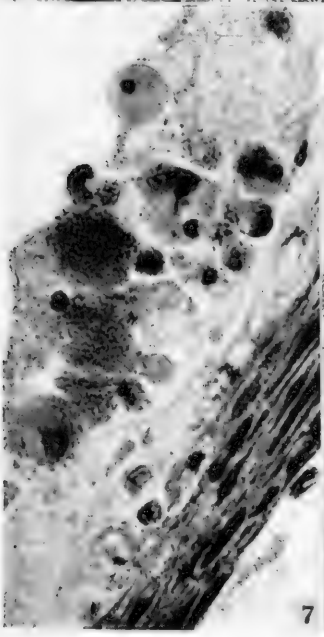

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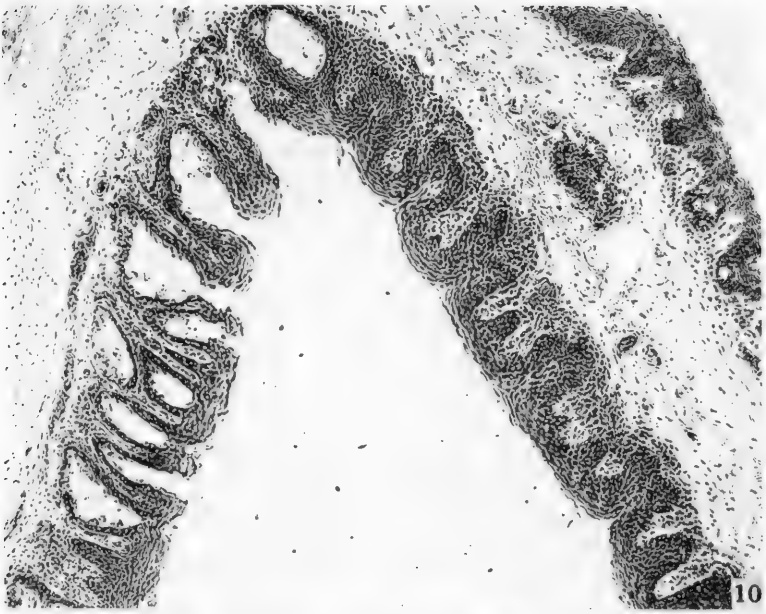

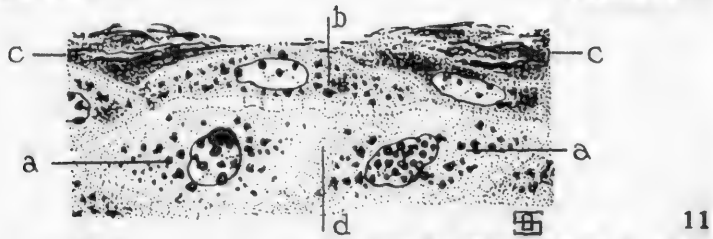

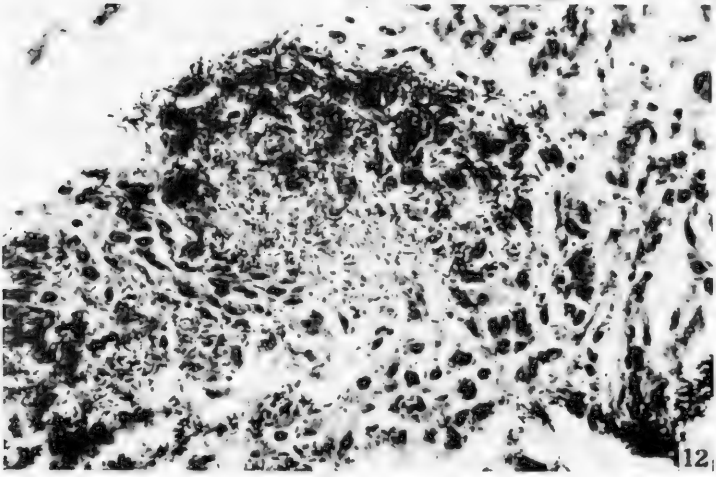

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STUDIES ON A-AVITAMINOSIS IN CHICKENS

II. Lesions of the Upper Alimentary Tract and Their Relation to Some Infectious Diseases

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Plates 24 and 25

(Received for publication, June 19, 1930)

In the previous paper[1] of this series the lesions in the respiratory tract caused by a lack of vitamin A in the food of chickens was considered, together with a discussion of the clinical findings and their relation to certain infectious diseases. Using the same material and methods, it is the purpose now to deal in the same way with the lesions of the mouth, palate, esophagus, crop, and their associated glands.

Gross Pathology

Because of the close connection between the nasal cavity and the mouth, the seromucous discharge in the former overflows regularly through the cleft palate and produces a clinical and anatomical picture which cannot be differentiated from that found in coryza contagiosa. As secondary invaders appear this exudate becomes transformed into white or slightly yellowish caseous masses which may at times completely plug the cleft palate. In addition, in the region of these nasal plugs and also on the roof of the mouth but seldom in the esophagus, fine thin membranes appear which together with the nasal plug produce a picture which may be easily confused with that of fowl-pox. In A-avitaminosis, however, these membranes may not always be present and when they are they usually are limited to the cleft palate and its adjacent epithelium. They are easily removed leaving no bleeding ulceration, thus differing from fowl-pox.

Early in A-avitaminosis there appear small white or yellowish pustule-like lesions, varying from 0.5 to 2 mm. in diameter, in the region of the excretory ducts of the glands of the mouth (Text-figs. 1 and 2). When discrete they are round, but by confluence they may become longish. They are usually raised above the

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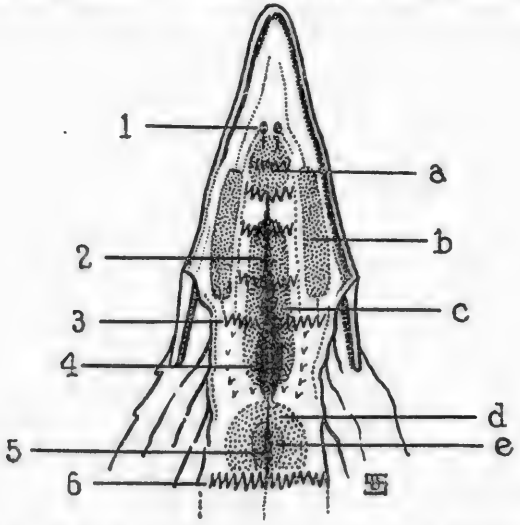
[1] Seifried, O., *J. Exp. Med.*, 1930, **52**, 519.

surface of the mucous membrane and often show a depression in the center (Fig. 1). Later small ulcers may appear at the site of these lesions and these ulcers may be surrounded by inflammatory products. These pustule-like lesions, with or without the inflammatory membranes, very closely resemble lesions of fowl-pox, and it is with great difficulty, if at all, that a differential diagnosis can be made by the macroscopical picture.

The mucosa of the esophagus and crop also shows pustule-like patches which may be even more prominent than those seen in the mouth, probably due to the



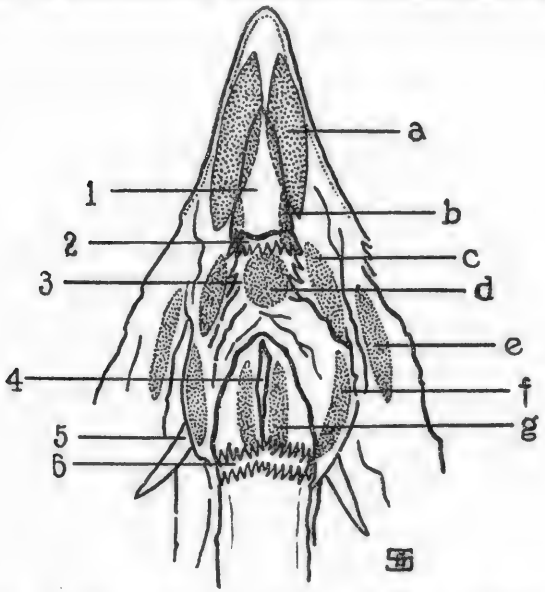
TEXT-FIG. 1. Roof of mouth. 1: excretory ducts of maxillary gland; 2: cleft palate, narrow part; 3: papillae of palate; 4: cleft palate, wide part; 5: infundibulum; 6: papillae of throat. a: glandula maxillaris; b: glandula palatina lateralis; c: glandula palatina medialis; d: glandula spenopterygoida; e: glandula tubaria.

peculiar structure of the glands in these regions (Fig. 1). They may be either localized in the upper part of the esophagus and surrounding the pharynx, or they may cover the mucous membrane of the esophagus throughout its entire length and in cases extend into the posterior part of the crop where the folds of the esophagus are continued. In the main portion of the proventriculus and throughout the intestines no gross lesions have been found. The nodules in the esophagus do not ulcerate as frequently as those in the mouth cavity, but they show a depressed minute opening in the center which corresponds to the excretory duct of the gland. In a few instances they are so numerous in the esophagus that when infected a type of membrane may be formed.

Histology

As noted above the lesions in the alimentary tract occur mainly in the glands. They have been studied in sections made of the roof of the mouth after decalcification.

Glands from the floor of the mouth, and in some instances from the roof, have been dissected out and sectioned in their shortest and longest diameters, while the



TEXT-FIG. 2. Floor of mouth. 1: tongue; 2: papillae of tongue; 3: base of tongue; 4: entrance to larynx; 5: bone of tongue; 6: papillae of larynx. a: glandula submaxillaris anterior; b: glandula lingualis anterior; c: glandula submaxillaris oromedialis; d: glandula lingualis posterior; e: glandula submaxillaris caudolateralis; f: glandula submaxillaris intermedia; g: glandula cricoarytenoidea.

glands of the tongue, pharynx and esophagus have been sectioned *in situ*. The lesions in the glands are essentially the same as those found in the respiratory tract and described in the previous paper. There are, however, some peculiarities which probably depend upon the special structure of the glands. As a rule the early lesions in these glands are not found in the acini but in the collecting spaces and ducts. Lesions may be well marked in the maxillary and submaxillary glands before they appear in the nasal passages, sinuses, trachea or bronchi. Fig. 2 shows

an early lesion of the submaxillary gland in which there is a marked proliferation and degeneration of the original epithelium. At the same time new stratified epithelium appears beneath or in place of the original epithelium. These early lesions may not be very characteristic but as the process is advanced typical pictures occur, as shown in Fig. 3 of the maxillary gland where in the collecting space is found a focus of squamous stratified keratinizing epithelium. In the acini the high mucus-secreting epithelium has begun to degenerate and atrophy and in a few instances there has been a replacement by stratified keratinizing cells. These changes in the acini have never been as pronounced as in the collecting spaces. Fig. 4, a cross section through the glandula lingualis, shows a well marked stage in the process with newly formed stratified epithelium and early keratinization. This newly formed epithelium may be found in isolated islands or in larger foci beneath or in place of the original epithelium lying directly upon the connective tissue. It seems probable that this newly formed tissue is favored in its growth by the abundant supply of capillaries from the surrounding connective tissue. The collecting spaces become filled with degenerated cells, masses of mucus (Fig. 4), and in some cases inflammatory products. This accumulation is undoubtedly in large part due to the lesions in the excretory ducts of the glands, as it is here that the oldest and most pronounced changes have been found. The epithelium of the mucous membrane of the mouth seems to extend into the duct, which is partially filled with the new stratified epithelium, and causes a more or less complete blocking. As a result the glands become dilated and frequently show evidences of bacterial infection, the latter being especially marked in the duct. Since this is an early lesion the gland may continue to secrete mucus for a time and this accumulates in the collecting space. Later desquamated cells from the newly formed stratified epithelium become numerous and the gland, which originally is a sac with invaginations, becomes smoothed out and distended. Finally this distended sac becomes completely filled with stratified keratinized epithelial cells, as shown in Fig. 5 which gives a picture of the final stage of the process. Such a picture is found more frequently in the glands of the tongue, palate and esophagus than in the compound glands of the cavity of the mouth.

The histological picture shows that early infections of the excretory ducts of the glands are relatively common. These infections are manifested by necrosis of the epithelial lining of the duct and of the surrounding mucous membrane, cellular infiltrations, and the presence of numerous bacteria (Figs. 6 and 7). Oftentimes it appears that the lumen of the duct is blocked, thus resulting in an accumulation of inflammatory products in the gland itself. At times the inflammatory process spreads from the mouth of the duct over the surrounding mucous membrane of the mouth (Fig. 8) or tongue, and more rarely over the mucous membrane of the esophagus.

In the submucosa signs of infection such as perivascular and diffuse infiltrations are frequently seen. In some sections through the tongue such infiltrations have been found even in the deeper layers of the muscularis, and in the ventral

portion of the vomer these lesions are particularly pronounced. In the esophagus and crop and sometimes in the proventriculus, the lesions in the glands are the same as those described in the mouth cavity but secondary bacterial infections are not as pronounced.

As pointed out above, the macroscopical lesions in the mouth may closely resemble lesions of fowl-pox. The histological difference between these two is quite striking and there is no difficulty in separating them from sections, but what part the process plays in the development of fowl-pox and other infections by breaking down the resistance of the mucous membranes is a problem that is well worth further consideration.

SUMMARY AND CONCLUSIONS

When fowls are placed on a diet lacking in vitamin A lesions appear in the upper alimentary tract which are confined largely to the mucous glands and their ducts. Histologically it is shown that the original epithelium becomes replaced by a stratified squamous keratinizing epithelium and that secondary infections are relatively common. The ducts of the glands may be blocked leading to distention with secretions and necrotic materials. These lesions macroscopically resemble very closely certain stages of fowl-pox and the two conditions can be separated only by histological examination. It is pointed out that these lesions produced by a lack of vitamin A may enable bacteria and other viruses to enter the body.

EXPLANATION OF PLATES

PLATE 24

FIG. 1. Pustule-like patches at the roof of the mouth, the base of the tongue and the mucous membrane of the esophagus. (Chicken 7, dead after 82 days on experimental diet.)

FIG. 2. Longitudinal section through the glandula submaxillaris of Chicken 11. × 55. Proliferation of the original epithelium lining the collecting space; first stage of process. Hematoxylin-eosin. (Killed after 56 days on experimental diet.)

FIG. 3. Cross section through the maxillary gland of Chicken 1. × 110. Small foci of stratified keratinizing epithelium in the collecting space; degeneration of the epithelium in the acini. Hematoxylin-eosin. (Killed after 30 days on experimental diet.)

FIG. 4. Cross section through the base of the tongue (glandula lingualis) of Chicken 9. × 80. Shows a more advanced stage of the process. The cells, which are to replace the original epithelium, form a continuous vascularized syncytium, lying directly upon the surrounding connective tissue. Beginning keratinization, desquamation and degeneration of the upper layers of the newly formed epithelium. Hematoxylin-eosin. (Dead after 75 days on experimental diet.)

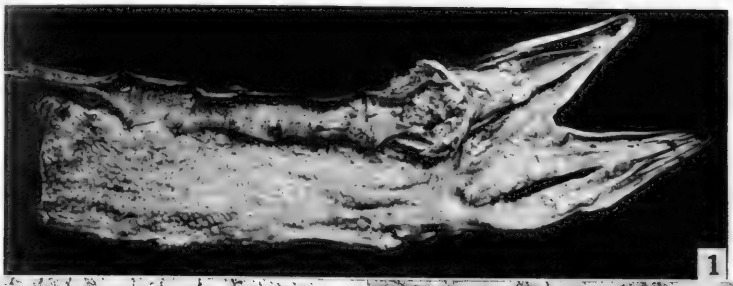
PLATE 25

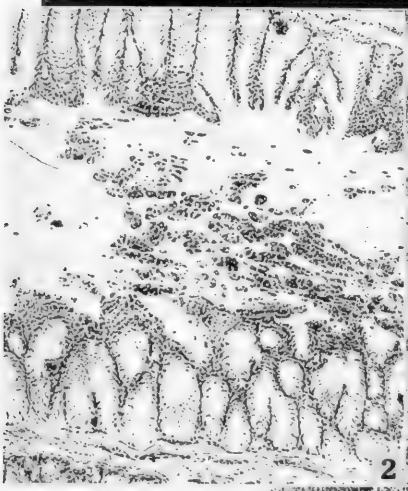
FIG. 5. Cross section through the base of tongue (glandula lingualis) of Chicken 6. × 50. Final stage of the entire process. The glands are completely filled with stratified, more or less keratinized, homogeneous masses. Extreme dilatation of the glands. van Gieson stain. (Dead after 87 days on experimental diet).

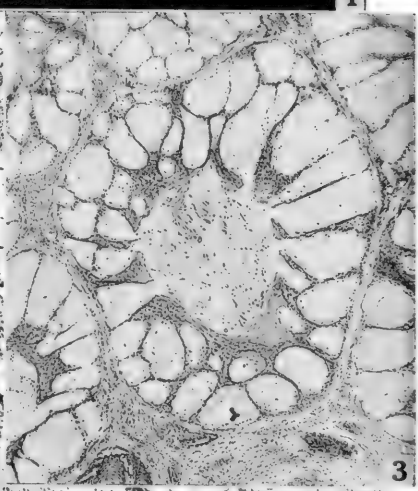
FIG. 6. Excretory duct of the glandula palatina medialis of Chicken 5. × 50. Bacterial infection followed by necrosis of the duct and its surroundings; accumulation of necrotic masses, mucus, inflammatory products and numerous bacteria. Giemsa stain. (Dead after 85 days on experimental diet.)

FIG. 7. Excretory duct of the lateral glandula palatina of Chicken 7. × 875. Invasion with numerous bacteria. Methylene blue stain. (Dead after 82 days on experimental diet.)

FIG. 8. Cross section through roof of mouth (glandula maxillaris) of Chicken 7. × 75. Thick membrane covering the mucous membrane, developing after secondary infection of the excretory ducts. Hematoxylin-eosin. (Dead after 82 days on experimental diet.)







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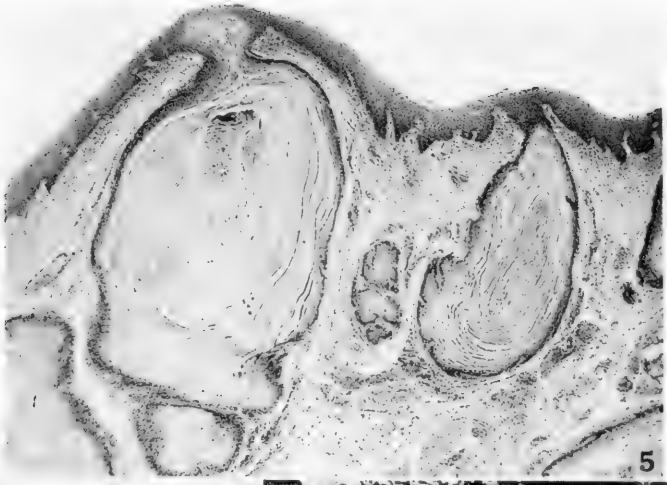

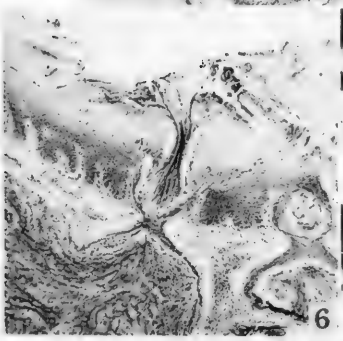

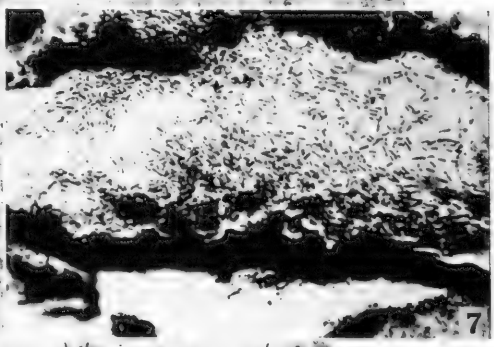

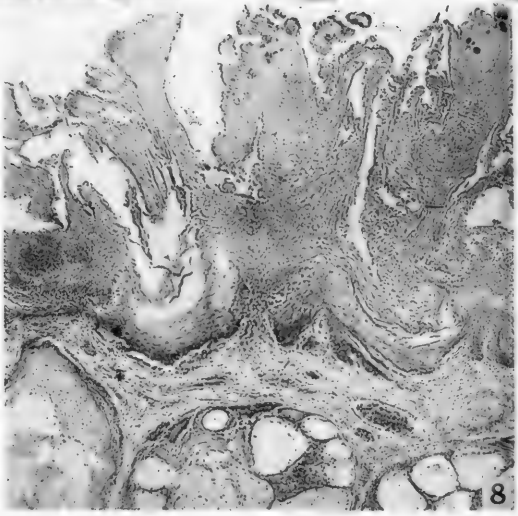

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THE DISTRIBUTION OF FRIEDLÄNDER'S BACILLI OF DIFFERENT TYPES

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(Received for publication, June 24, 1930)

In 1926 (1), a number of strains of Friedländer's bacillus were classified by the immunological reactions of agglutination, agglutinin adsorption and passive protection against infection, into three specific types, which were arbitrarily designated as Types A, B, and C. The strains falling into the specific types comprised the great majority of those studied, and the remaining cultures which were found to differ serologically from each other and from the specific types were included in a heterogeneous group called Group X. The distinctions in type were referable to the presence of a specific carbohydrate in the capsular material. The carbohydrate (2), or soluble specific substance, was shown to be chemically different for each of the three types.

Cultures of Friedländer's bacillus have been collected in the meantime in order to study the relative frequency and distribution of the organism of various immunological types.

EXPERIMENTAL

In the present survey a total of 80 strains have been isolated from a variety of disease conditions; in most instances the organisms were considered to be the inciting agent of the disease, in other cases secondary invaders. All the organisms studied were encapsulated, nonmotile, non-spore-bearing, Gram negative rods with the cultural characteristics usually attributed to Friedländer's bacillus. In each instance the serological type was determined by the agglutination of live young cultures (4 to 6 hours) in type-specific sera.

The fermentation of carbohydrates by the different strains was also studied to determine possible serological and biochemical relationships.

The Frequency of Occurrence of Friedländer's Bacilli of the Different Specific Types.—In Table I is given the relative number of strains of the different types isolated from disease. It is seen that of the 80 strains, about 75 per cent fall in one or other of the three specific types. To Type A, belong 42 of the strains (52 per cent); to Type B, 12 (15 per cent); to Type C, 7 (9 per cent). Group X includes 19 (24 per cent) of the cultures. It is interesting to compare these figures with those obtained from the original study (1) of 30 strains. At that time, it was found that 50 per cent of the strains were of Type A, 20 per cent of Type B, 10 per cent of Type C, and 20 per cent belonged in Group X.

The Incidence of the Various Specific Types in Different Diseases.—This is shown in Table II. Most of the strains studied were isolated from cases of pneumonia in man. Of the strains isolated from this

TABLE I

The Distribution of Specific Types of Friedländer's Bacillus

Total number of strains studied	Distribution by types			Group X
	Type A	Type B	Type C	
80	42	12	7	19

source about 75 per cent were of Type A. In the original study (1) it was found that about 70 per cent of the total strains associated with pneumonia belonged to Type A. Most of the Type A strains were isolated from human cases where the majority of Type B strains were isolated from animals. The organisms of Group X were obtained from a variety of conditions in animals and man.

The Specific Precipitin Reaction in the Urine in Pneumonia.—In a preceding paper (3) it was shown that rabbits experimentally infected with Friedländer's bacillus eliminate the soluble specific substance through the kidney, and that the urine of infected animals may give a precipitation reaction in the corresponding type-specific serum. Blake (4) first demonstrated the presence of a positive precipitin reaction in a case of pneumonia in man due to Friedländer's bacilli. Recently in a case of pneumonia in the Hospital of The Rockefeller Institute, due to Friedländer's bacillus, the opportunity was offered

for studying the type-specific precipitin reaction in the urine. On the day of admission, which was the second day of the disease, Friedländer's bacillus Type A was isolated both from the blood and sputum, and the presence of the soluble specific substance was demonstrated in the urine by a type-specific precipitation reaction which was positive even when the urine was diluted sixteen times. On the following

TABLE II

The Incidence of the Various Types of Friedländer's Bacillus in Different Diseases

Type	Human pneumonia	Throat	Liver abscess	Animal source	Miscellaneous
Type A (42 strains)	33	3	1	Guinea pig—2 (not pneumonia) (fatal abscess)	Cystitis—1 Adenoid tissue—2
Type B (12 strains)	2	1	1	Guinea-pig—5 (pneumonia) Mare—2 (G. U. infections)	Feces—1 (colitis)
Type C (7 strains)	3	0	0		Antrum—1 (sinusitis) Nose—1 (sinusitis) Unknown—2
Group X (19 strains)	7	2	1	Guinea-pigs—2 (not pneumonia) (fatal abscess)	Sputum—2 (not pneumonia) Cystitis—3 Lung abscess—1 Feces—1 (Pellagra)

day the disease terminated fatally and this day the precipitation reaction was positive when the urine was diluted 1:64.

The Fermentation of Carbohydrates by Friedländer's Bacilli of the Different Types.—The fermentation reactions of bacteria of the *Encapsulatus* group have been studied by a number of workers, but the results have been contradictory and confusing. While certain authors have found that Friedländer's bacillus ferments lactose with the formation of acid and gas (5, 6, 7, 8) or of acid alone (9, 10, 11, 12), other writers (13, 14, 15) have observed that it does not ferment

lactose. Still other authors (16, 17, 18, 19) state that different strains may vary in their ability to ferment lactose, and it is the opinion of these authors that the fermentation reactions of the bacteria of the

TABLE III

The Fermentation Reactions of Friedländer's Bacillus of the Different Specific Types

Friedländer bacilli		Reactions in				
Type	Number of strains	Dextrose	Lactose	Sucrose	Maltose	Mannitol
A	30	AG	AG	AG	AG	AG
B	8					
C	3					
Group X	8					
A	9					
B	None	AG	Negative	AG	AG	AG
C	1					
Group X	1					
A	2					
B	2	A	A	A	A	A
C	None					
X	5					
A	4					
B	None	A	Negative	A	A	A
C	None					
X	None					
X	1	AG	AG	Negative	AG	Negative
X	1	A	Negative	Negative	Negative	Negative
X	1	A	Negative	Negative	AG	Negative

A indicates presence of acid.
AG indicates presence of acid and gas.
Negative indicates absence of acid and gas.

Encapsulatus group are too inconstant to be of value in classification or identification.

It seemed, however, important to study again this question with reference to the specific types of organism employed in making the tests.

Five carbohydrates (dextrose, lactose, sucrose, maltose and mannitol) were employed, since earlier observations indicated that these five offered the greatest possibilities for use in differentiation. Samples of meat extract broth containing 1 per cent of the respective carbohydrates were inoculated, and the final readings were made after an incubation period of 7 days.

The results of this study are given in Table III. It is seen that the majority of the strains ferment all 5 carbohydrates with the formation of acid and gas. There is, however, a great variability in the fermentative activities of the different strains, and it is obvious that a classification of Friedländer's bacilli on the basis of fermentation activity is of little value. Furthermore, the fermentation reactions do not aid in the identification of the specific types. It is interesting to note, nevertheless, that of 15 strains which form neither acid nor gas in lactose, 13 are of Type A. In other words, the inability to ferment lactose is most characteristic of Type A cultures. Of further interest is the fact that the strains of Group X show the greatest variability in the fermentation of carbohydrates.

DISCUSSION

The present communication records the distribution of Friedländer's bacilli of the different specific types in disease. It was found that most of the Type A strains are obtained from human cases and the majority of them from cases of pneumonia. On the other hand, the greatest number of strains of Type B are derived from animal sources, while Group X contains organisms isolated from the greatest variety of diseases. In this connection, it should be pointed out that in confirming the existence of specific types among the Friedländer bacilli, Edwards (18, 19) characterizes his Type I (corresponding to Type B) as the animal type, and Type II (corresponding to Type A) as the human type. Edwards (19) also shows that certain strains of *Bacterium aerogenes* are serologically identical with Friedländer's bacilli. This is not surprising, however, when it is recalled that Avery, Heidelberger and Goebel (20) had previously demonstrated the immunological similarity of Type B Friedländer's bacillus to the more widely remote species, Pneumococcus Type II.

It has been possible to confirm Blake's observations on the occur-

rence of a specific precipitin reaction in the urine during the course of pneumonia due to Friedländer's bacillus. Dochez and Avery (21), also demonstrated during their original studies on the soluble specific substance of Pneumococcus that the specific urine precipitation tests in immune sera offered a procedure of diagnostic as well as prognostic importance.

A study of the fermentation reactions of Friedländer's bacillus confirms an earlier opinion (17) that a classification based on these reactions possesses little value. Fitzgerald (16) previously and Edwards (18, 19) more recently reached similar conclusions. In the present study, it was not possible to show any constant relationship between serological reactions and biochemical activity as measured by the fermentation of carbohydrates, although it was found that among the strains of all types there is a much greater tendency for Type A strains not to form acid or gas from lactose.

SUMMARY AND CONCLUSIONS

In a study of the distribution of the specific types of Friedländer's bacillus, it is shown that: (1) Of 80 strains 52 per cent belong to Type A, 15 per cent to Type B, 9 per cent to Type C, and 24 per cent to Group X. (2) Type A contains for the most part strains derived from diseases of man and more than 70 per cent are associated with pneumonia in man. (3) Type B includes the greatest number of strains from animal sources, while the heterogeneous strains comprising Group X come from the greatest variety of diseases.

It was demonstrated that in a patient suffering with pneumonia due to Friedländer's bacillus (Type A), a specific precipitin reaction of the urine occurred in the corresponding (Type A) immune serum.

A study of the sugar fermentation reactions of Friedländer's bacillus shows that (1) there is no correlation between serological type and fermentative activity; (2) the fermentation reactions are variable and therefore not reliable for distinguishing Friedländer's bacillus from closely allied organisms; (3) the strains of Group X show the greatest variation in fermentation, and (4) of 15 strains unable to ferment lactose, 13 belong to Type A.

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STUDIES OF DISEASES OF THE LYMPHOID AND MYELOID TISSUES. I.

The Chemical Metabolism of Normal and Pathological Lymph Nodes*

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(Received for publication, June 25, 1930)

Warburg and his associates, Posener and Negelein (1924), have shown that within certain limits and with certain exceptions carcinomatous tissue has a unique and characteristic metabolism. They and Warburg (1925) found that normal tissues have a relatively high rate of oxygen consumption and a low aerobic glycolysis. Embryonic tissue has high respiration and, under anaerobic conditions, a high glycolysis, but it has only a slight destruction of sugar under aerobic conditions. Neoplastic tissue, on the other hand, has a rather low respiration rate and a high glycolysis under both aerobic and anaerobic conditions. These broad generalizations have been corroborated by Murphy and Hawkins (1925), Rona and Deutsch (1926), and others. The subject is more fully reviewed by Jackson (1929). Neither glycolysis nor oxygen consumption, however, proved to be an infallible guide to the nature of a given living tissue, so that Warburg (1927) resorted to a new value by which to differentiate neoplastic from normal tissue. He points out that when the Pasteur reaction functions at its maximum, the formation of exactly two molecules of lactic acid is suppressed when one molecule of oxygen is consumed, and on this basis he suggests a new value "U," which may be regarded as the

* The expenses of this work were in part defrayed by Mr. Edward Mallinckrodt, and in part by the DeLamar Mobile Research Fund, Harvard University.

theoretical aerobic glycolytic rate. This value U he finds rather constantly negative for normal tissue, in the vicinity of zero for embryonic tissue, and strongly positive for carcinomata.

In man the lymph nodes are subject to various pathological processes including what, for lack of a better term, we may call the malignant lymphomata, a classification which is used in this paper to include lymphosarcoma, Hodgkin's disease, reticulum cell sarcoma and lymphatic leukemia. Pathologists are not entirely agreed as to the nature of these rather varied, but at the same time similar, pathological processes. Some regard them as neoplasms, others as infectious granulomata. Other pathologists again would class some types in one group, some in the other. In this paper we are placing Hodgkin's disease, as defined below, by itself for comparison with the other types of lymphomata.

As a part of a general investigation of this group of diseases it occurred to us that a study of the chemical metabolism of pathological lymph nodes might throw some light on their fundamental nature. The metabolic processes might, we hoped, afford evidence as to whether various types of malignant lymphomata were neoplastic or infectious in origin. This paper deals with the results of the investigation of normal and pathological lymph nodes and primary tumors not involving lymph nodes, from 71 patients, and is the first of a series of papers dealing with various aspects of malignant lymphomata.

Methods

As soon as received from surgical operation, the tissue was cut into thin sections, averaging 0.3 mm. in thickness, by a specially devised knife consisting of two safety razor blades held parallel, but slightly apart, in a suitable metal holder provided with adjustments for aligning the blades. All such sections were immediately immersed in sterile, normal horse serum, at room temperature, preparatory to their transfer to the metabolism chambers. Sterile cultures of the lymph nodes in this horse serum lived as long and as well as in the patient's own serum. Paraffine sections were made of all lymph nodes examined.

The metabolism was carried out in all instances in a modified Thünburg respiration apparatus. Briefly this consisted of two small vials connected one with another by means of a long, very fine capillary tube containing a kerosene drop to serve as a direct indicator of the oxygen consumption. The tissue to be metabolized was floated in buffered normal horse serum in an inner chamber. The moat about this inner chamber contained 2 per cent NaOH which served to absorb all

carbon dioxide formed. The anaerobic glycolysis was determined in a small vial containing horse serum saturated with pure nitrogen which had been passed over red hot copper to free it from traces of oxygen. The serum used in the anaerobic experiments was first subjected to a vacuum to rid it of air and subsequently saturated with pure nitrogen. This anaerobic chamber was attached to the stand supporting the Thünburg apparatus. The whole apparatus, aerobic and anaerobic alike, was immersed in a constant temperature water bath at 37.5°C. and kept agitated by a suitable motor. All apparatus coming in contact with the tissue was chemically clean. That the possible presence of bacteria did not affect the results was evident from several specially devised experiments and also from the constancy, from period to period, of the tissue metabolism, as evidenced by the oxygen consumption. Following each experiment the tissue was washed free of serum, dried at 100°C., and weighed. From 5 to 25 mg. tissue was used. All weights refer to dry tissue.

Oxygen consumption was read directly on a calibrated scale attached to the capillary. Readings were taken every 5 minutes. The results are expressed in c. mm. per mg. hour. Glycolysis, or more accurately, sugar destruction during the experimental period was determined by the familiar method of Folin (1929). The figures given in the text are those actually observed, except that in those tumors in which there was considerable connective tissue stroma a suitable allowance was made as this tissue has a metabolism entirely negligible compared to that of the more active cells composing the rest of the tumor. The amount of stroma present was carefully estimated by two independent observers from paraffine sections. The observed metabolic figures were then multiplied by the appropriate factors. In only 14 of the 71 cases was the amount of stroma increased sufficiently to warrant the application of a correctional factor.

Experimental Results

Normal Nodes.—Four normal nodes were examined. The average aerobic glycolysis was 0.020 mg., the average anaerobic glycolysis 0.054 mg., and the average oxygen consumption 5.7 c. mm. In this small series of cases there was a marked uniformity of the results. The average value U was 4.1, a figure entirely consistent with that found by Warburg for normal lymphoid tissue, but slightly higher than that found in most normal tissues elsewhere in the body which, as has already been stated, usually have a negative U. These figures can probably be taken as representative of the normal lymph node, and must be used as a determining value for further correlations.

Lymphoma.—Under this heading we here include lymphosarcoma, lymphatic leukemia, lymphocytoma, lymphoblastoma, and reticulum

cell sarcoma, but not Hodgkin's disease. There were studied metabolically nodes from 14 such cases. In contradistinction to the normal nodes there was found to be a marked variation in the different factors making up the metabolism in these pathological cases. The average aerobic glycolysis was 0.063 mg., over three times that found in the normal series. Three cases had an aerobic glycolysis above 0.083 mg., and all of these cases were rapidly fatal, while in two cases the rate fell below 0.035 mg., and these also were highly malignant from a clinical point of view. No correlation, therefore, could be found between degree of malignancy and the rate of aerobic glycolysis. The nodes from case S-29-662 and those from case S-29-1574 gave almost identical figures for aerobic glycolysis, yet the former case is alive and well a year and a half later, while the latter case died in a few months. The average anaerobic glycolysis was 0.092 mg. One, a case of rapidly advancing lymphosarcoma (S-29-236), showed the extremely high figure of 0.206 mg., while another, a case of lymphosarcoma involving the stomach (S-29-1585), had a low figure of 0.047 mg. Again, no correlation can be traced. The oxygen consumption averaged 5.5 c. mm. per mg. hour with such extreme variations as 0.7 and 12.6, yet there was no apparent relation between the metabolic rates and either the pathological or the clinical findings. The value U averaged 11.8, thus placing these tumors tentatively in the neoplastic class. But again extreme variations occurred, as witness case S-29-100 A, a rapidly advancing and rapidly fatal lymphoma with metastases to bone which had a U value of -1.7, and, on the other hand, S-29-236, a lymphosarcoma with a U of $+47$ and fatal in approximately the same time as the first case. Again case S-29-662 had a U value well above the general average for this class and well within the limits set by Warburg for malignancy, yet the case responded in a remarkable way to high voltage X-ray therapy and has remained well over a period of nearly 2 years.

Hodgkin's Disease.—Eighteen cases of typical Hodgkin's disease were studied. We class as Hodgkin's disease those nodes which show the typical multi-nucleated giant cells described by Dorothy Reed and by Sternberg. The tumor in some of the cases was very cellular, while in others it was sclerosed. The average aerobic glycolysis was 0.052 mg., slightly lower than the corresponding figure for all other types of lymphoma. There was a remarkable tendency for the figures to vary

about the average within narrow limits, the lowest being 0.030 mg. and the highest 0.067 mg. The average oxygen consumption was 5.8 c. mm. The anaerobic glycolytic rate was 0.074 mg., again slightly lower than that in the other lymphomata. The value U averaged 7.3, slightly above the corresponding value for normal nodes but definitely below that for tuberculous nodes which was 10.3. From this it might be inferred that Hodgkin's disease was infectious in nature, or at least not neoplastic, but it should be remembered that the lymphosarcoma S-29-100A had a value U of only —1.7, and another lymphosarcoma infiltrating the stomach and the parotid had a value of 3.4. On the other hand, a case of sclerosing Hodgkin's disease (S-29-1152), responding well to X-ray therapy, showed in the node a value U of 19.6, while another case, histologically similar and clinically rapidly fatal (S-29-1556) had a low value of 0.9. So it must be recognized that no very clear evidence is obtained as to the fundamental nature of the lesions from these data.

Tuberculosis.—Four cases of the cellular type of tuberculosis were studied. Necrotic or very fibrous nodes were excluded for obvious reasons. Here it was found that the average aerobic glycolysis was 0.055 mg., and the average anaerobic 0.090 mg. The oxygen consumption ran high, averaging 8.2 c. mm., while the U averaged 10.3. Nye and Parker (1930) have produced in animals lesions consisting of closely packed mononuclear cells identical with, or very similar to, those found in cellular tuberculosis. The lesion is particularly prominent and particularly pure in the lung, and examination of the involved lungs from these animals showed that they had an aerobic glycolysis of 0.05 mg. and anaerobic of 0.09 mg., a U of 15. The similarity between these figures and those of human tuberculosis is obvious, and the figures indicate that the type cell rather than the etiological agent or the fundamental nature of the disease is responsible for the character of the metabolism. If one attempts to classify tuberculosis on the basis of Warburg's figures one is forced to admit that here again is an obvious exception to his general rule; for the observed figures alone would indicate rather sharply that the process is neoplastic.

Carcinoma.—Thirteen cases of carcinomata involving lymph nodes were examined. Here again, as in the case of Hodgkin's disease, due allowance was made, when necessary, for the presence of large amounts of physiologically inactive connective tissue. The average aerobic

glycolysis was 0.088 mg., a figure far higher than any other tissue previously examined, but rates as low as 0.032 mg. (S-29-1042) were found in a slowly growing, highly differentiated epidermoid carcinoma. Five cases had an aerobic glycolysis above 0.100 mg., and 10 were above 0.060 mg. The average anaerobic rate was 0.142 mg., but varied considerably. With one exception, case S-29-1042 again, all were above 0.068 mg., and six cases yielded figures which were above 0.150 mg. Oxygen consumption was consistently low, averaging 4.1 c. mm. As was to be expected from Warburg's work, the value U was very high, averaging 24, well over twice that for lymphoma and entirely in confirmation of Warburg's findings. The highest U value was 40.4 in a rapidly growing, highly malignant epidermoid carcinoma (S-28-2468). There seemed, indeed, to be some general correspondence between the value U and the degree of malignancy. Thus, S-29-1049, S-28-168J, and S-28-2468 all had a U value above 35 and were all highly malignant, while S-28-2524 and S-29-1042 were relatively benign, slow growing tumors, and each had a value below 11. But the rule was by no means invariable. Roughly the same crude parallelism could be traced between malignancy and anaerobic glycolysis, though again there were certain exceptions.

Sarcoma.—In all, thirteen cases of sarcoma were studied. In most cases the tumors investigated were primary foci and not metastatic in lymph nodes. The metabolism was striking and interesting, in that all figures were low. The average aerobic glycolysis was 0.017 mg., with comparatively little variation except in S-29-1803, where the figure was 0.036 mg. The average anaerobic glucose destruction was 0.037 mg., again varying closely about the mean. The U value averaged only 4.9 and the oxygen consumption only 2.4 c. mm. It is be noted that all these figures are far below those of carcinoma. To be sure, S-28-1803 with a U of 12.6 comes into the malignant class, but the majority fell far short of it and yet they were all highly malignant tumors. S-29-2965, a melanotic sarcoma, had an anaerobic glycolytic rate of but 0.056 mg. with a U of but 7.4, yet it was one of the most rapidly growing tumors ever seen in this laboratory. The conclusion is obvious. Sarcomata do not appear to behave metabolically in the same manner as do carcinomata, and from their relatively low metabolic rates it is difficult to understand wherefrom they derive the great energy which must be required to favor their growth and

advance. If one figures, according to the method of Hawkins (1925) the energy requirements of a rapidly growing carcinoma such as S-29-1049, one finds that it produces anaerobically 0.093 calories per mg. dry weight per hour, aerobically 0.206 calories. The same calculations applied to such an extremely rapidly growing sarcoma as S-29-2965 show that anaerobically it produces but 0.020 calories and aerobically only 0.022 calories. Again the distinction, metabolically speaking, between carcinomata and sarcomata is obvious. They do not behave in the same manner. It should be noted, however, that even carcinoma itself does not appear in such experiments as these to expend much energy in growing. Aerobically the average energy output of these series of carcinomata was 0.033 calories per mg. hour, anaerobically 0.054 calories. The respective averages for sarcomata were 0.018 and 0.014 and for benign tumors 0.049 and 0.006.

Benign Tumors.—Three benign tumors were investigated. As was to be expected, their metabolism was low, the aerobic glycolysis being 0.010 mg., and the anaerobic 0.017 mg., and the U 3.9—figures, it should be noted, not far from those of the highly malignant sarcoma. Their oxygen consumption averaged 2.4 c. mm.

The degree of differentiation of various cells seemed rather definitely connected with the degree of difference between the aerobic and anaerobic glycolysis.

For instance, Cases 9, 19, 33, 38, and 40 were tumors (carcinoma, lymphoma, etc.) composed of undifferentiated cells, and their percentage difference was only 1 per cent, the highest being 8 per cent. On the contrary, Cases 1, 8, 11, 12, 15, 16, 21, 30, 37, 41, 42, and 78 were all tumors composed of well differentiated cells, and the average difference was 167 per cent. Case 12 (S-28-2431) constituted an exception in that it was a highly differentiated tumor (epidermoid carcinoma) with a split of only 11 per cent. No correspondence was found to exist between the oxygen consumption and the maturity of the cells, though Glover, Daland and Schmitz (1930) have found with white blood cells of both the myeloid and lymphoid series a definite increase of oxygen consumption with increasing maturity. This they found to hold true only when the cells were studied in whole blood but not when they were suspended in Ringer's solution. For various reasons they believed that whole blood gave more accurate information. Exceptions to this general rule (the greater the difference between

aerobic and anaerobic glycolysis the greater the degree of cell differentiation) occurred in almost all the sarcomas, as witness S-29-1913, a highly differentiated tumor with a split of 0, and S-28-2721, an undifferentiated cell tumor with a split of 127 per cent.

DISCUSSION

It was hoped that evidence might be thrown on the question as to whether the malignant lymphomata are of infectious or neoplastic origin from this study of the aerobic and anaerobic glycolysis and the oxygen consumption of 71 lymph nodes and tumors. Careful comparisons have been made between the microscopic pathology and the metabolism. The various metabolic rates were found to vary over so large a range that few generalizations can be made. There was, in general, a steady rise in both the aerobic and anaerobic glycolysis from the normal nodes through Hodgkin's disease, the lymphoblastomata and tuberculous nodes to carcinomata. The sarcomata and benign tumors had very much lower rates. Their glycolysis, indeed, is not even of the same order of magnitude as that of the carcinomata. Much overlapping existed, however, so that from the metabolism alone one can not infallibly predict in which class the tissue will fall. Some lymphomata, for instance, had metabolic rates greater than those of some cancers. The oxygen consumpton seemed to bear little or no relation to the pathological picture. It was rather consistently low in carcinoma and tended to be high in tuberculosis and some cases of Hodgkin's disease. The value U was over twice as high in the carcinomata as in any other pathological class, but here again overlapping existed to a considerable extent so that a diagnosis of neoplasm could not be made on this basis alone. The U value for sarcoma was definitely low, in no way approaching that of other malignant disease. The high metabolic rates found for tuberculous nodes indicated very clearly that the type of cell, rather than the nature of the underlying process, determined the character of the chemical changes. This fact was further borne out by the figures found for the rabbit lungs filled with mononuclears. Neither speed of growth nor degree of malignancy could be predicted from the glycolysis or oxygen consumption.

The metabolic processes in the sarcomata were of unusual interest. The figures of our thirteen cases approached those of benign tumors and were in no way similar to those of carcinomata. Yet some of these

sarcomata were extremely malignant and rapidly growing. The number studied is small and it is quite possible that further investigations will show that some human sarcomata have as high or even higher metabolic rates than do carcinomata of equal malignancy. Such is not our finding at present and further observations on sarcomata are in progress.

Such metabolic rates as are reported here represent the chemical activity of the tissue during a relatively brief period of tissue existence. They do not necessarily represent the requirements of growth. One of the essential characteristics of neoplastic tissues is their unlimited, chaotic and purposeless growth. To study the chemistry of growth rather than that of mere existence one must determine the metabolic rates of tissues over a long period of time in tissue cultures. This is being done as a continuance of the present study.

CONCLUSIONS

From a study of the metabolism of 71 lymph nodes and tumors one may conclude:

1. The nature of a tumor can not be predicted from the metabolism because too much overlapping of metabolic rates exists between the pathological groups.

2. There is no evidence metabolically one way or another as to whether malignant lymphomata of any type should be classed as neoplastic or as infectious processes.

3. The degree of cell differentiation can in most cases be foretold by the percentage difference between the aerobic and the anaerobic glycolysis. The greater the differentiation the greater the percentage difference. Sarcomata in general constitute an exception to this rule.

4. The degree of malignancy in carcinoma, but not in other tumors, can, with certain exceptions, be predicted from the height of the value U.

5. Human sarcomata appear to have a metabolism far more closely comparable to that of benign tumors than to that of carcinomata. They do not behave as malignant tumors under the Warburg classification. Their energy requirements are not of the same order as those of carcinoma.

6. One can not from the value U or from the glycolytic rates predict whether or not a tissue should be classed as neoplastic.

7. Warburg's findings for carcinomata are confirmed and amplified.

TABLE I

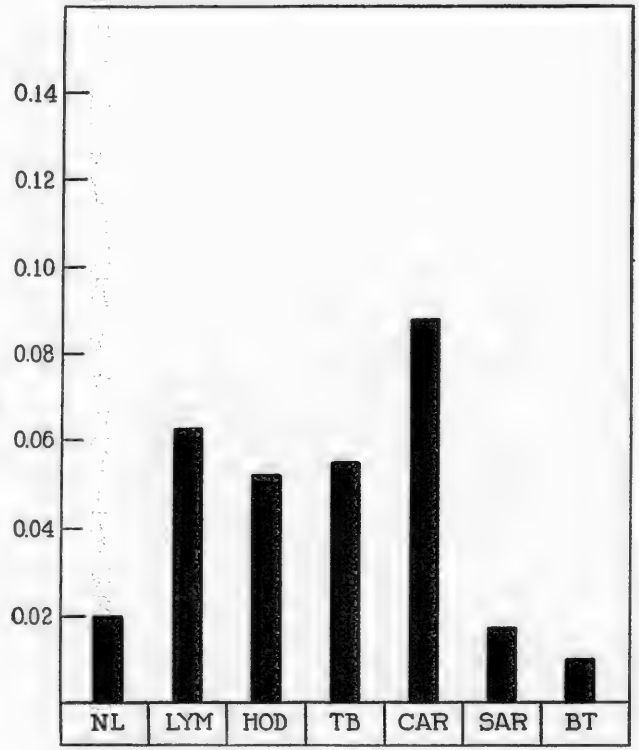
No.	Path. No.	Per cent active tissue	*Aerobic glycolysis	*Anaerobic glycolysis	†Oxygen consumption	"U"	Aerobic-anaerobic split per cent	Diagnosis
1	28-168J	50	0.116	0.172	3.2	36.6	48	Carcinoma
2	28-157R	100	0.071	0.117	6.4	16.4	65	Rabbit lung mononuclears
3	28-158R	100	0.050	0.106	5.2	16.1	110	" "
4	28-159R	100	0.051	0.100	5.1	15.0	96	" "
5	28-1686	100	0.034	0.037	2.0	4.5	9	Lymphoma
6	28-1804	100	0.066	0.082	4.4	11.7	24	Hodgkin's
7	28-2093	100	0.041	0.062	4.6	5.8	46	Hodgkin's
8	28-2347	100	0.009	0.014	1.0	2.5	56	Carcinoid
9	28-2348	100	0.069	0.069	5.0	7.5	0	Hodgkin's
10	28-2370	100	0.024	0.033	0.6	6.5	37	Normal node
11	28-2385	100	0.017	0.028	2.3	2.4	65	Arachnoidal fibroblastoma
12	28-2431	50	0.140	0.156	6.4	20.6	11	Carcinoma
13	28-2454	100	0.096	0.126	9.5	12.2	31	Tuberculosis
14	28-2467	100	0.055	0.082	1.8	17.9	51	Hodgkin's
15	28-2468	60	0.087	0.198	4.5	40.4	127	Carcinoma
16	28-2475	30	0.114	0.138	2.1	30.3	21	Carcinoma
17	28-2494	100	0.078	0.100	7.5	10.0	28	Hodgkin's
18	28-2516	100	0.040	0.069	4.4	8.4	73	Tuberculosis
19	28-2524	30	0.096	0.096	8.4	7.2	0	Carcinoma
20	28-2554	100	0.026	0.044	4.8	1.4	70	Endothelioma
21	28-2596	100	0.028	0.051	3.7	5.3	82	Normal nodes
22	28-2611	60	0.054	0.075	7.8	3.1	40	Reticulum cell sarcoma
23	28-2625	100	0.050	0.090	5.5	11.5	80	Hodgkin's
24	28-2665	100	0.067	0.096	6.4	11.2	43	Hodgkin's
25	28-2703	100	0.049	0.056	5.3	3.6	16	Hodgkin's
26	28-2707	100	0.019	0.040	2.1	5.8	110	Meningioma
27	28-2721	100	0.014	0.032	1.4	5.2	127	Sarcoma
28	28-2758	50	0.070	0.123	7.0	16.8	61	Carcinoma
29	28-2775	100	0.014	0.024	2.8	0.4	71	Fibrosarcoma
30	28-2795	100	0.056	0.078	5.1	9.3	39	Reticulum cell sarcoma
31	28-2917	75	0.076	0.082	3.7	13.6	8	Carcinoma
32	28-2931	100	0.077	0.106	6.8	11.7	35	Hodgkin's
33	28-3075	100	0.019	0.042	2.4	5.7	121	Normal node

* Expressed in mg. per 10 mg. dry weight per hour.

† " " c. mm. per mg. dry weight per hour.

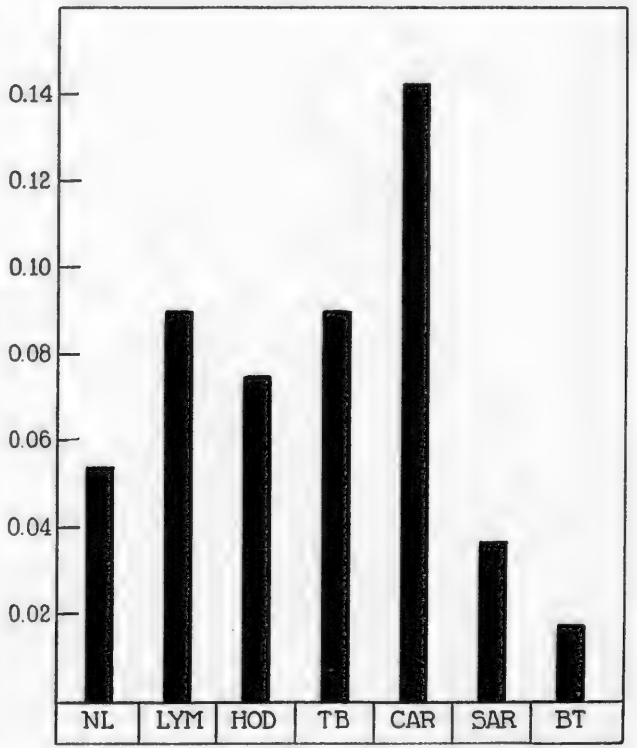
TABLE I—*Concluded*

No.	Path. No.	Per cent active tissue	*Aerobic glycolysis	*Anaerobic glycolysis	†Oxygen consumption	"U"	Aerobic-anaerobic split per cent	Diagnosis
34	29-100A	100	0.090	0.093	12.5	−1.7	3	Lymphoma
35	29-236	100	0.083	0.206	1.8	47.9	149	Lymphoma
36	29-266	20	0.115	0.115	4.5	17.8	0	Carcinoma
37	29-365	100	0.059	0.075	2.5	13.8	27	Lymphoma
38	29-399	100	0.044	0.045	0.7	9.8	2	Lymphoma
39	29-585	100	0.022	0.095	1.6	20.5	330	Lymphoma
40	29-628	100	0.001	0.012	4.4	−8.5	1000	Glioma
41	29-639	100	0.010	0.090	11.8	−1.1	800	Normal node
42	29-640	100	0.030	0.054	4.7	4.1	64	Hodgkin's
43	29-662	100	0.077	0.136	8.6	16.8	77	Lymphoma
44	29-1027	100	0.019	0.074	4.0	10.5	290	Tuberculosis
45	29-1042	75	0.032	0.048	2.0	10.0	50	Carcinoma
46	29-1049	50	0.150	0.250	29.5	35.5	67	Carcinoma
47	29-1152	100	0.047	0.100	2.7	19.6	112	Hodgkin's
48	29-1279	100	0.100	0.100	6.4	12.2	0	Lymphoma
49	29-1556	100	0.025	0.031	3.4	0.9	32	Hodgkin's
50	29-1574	100	0.071	0.071	6.4	4.9	0	Lymphoma
51	29-1585	100	0.047	0.047	4.8	3.4	0	Lymphoma
52	29-1754	100	0.068	0.079	9.7	0.3	14	Hodgkin's
53	29-1778	100	0.059	0.105	2.3	20.9	78	Carcinoma
54	29-1802	100	0.047	0.057	4.3	5.6	21	Hodgkin's
55	27-1803	100	0.036	0.077	3.3	12.6	114	Fibrosarcoma
56	29-1834	100	0.043	0.068	8.9	−0.9	58	Hodgkin's
57	29-1913	100	0.016	0.016	1.2	2.9	0	Fibrosarcoma
58	29-2066	33	0.042	0.168	3.9	34.2	300	Carcinoma
59	29-2309	20	0.050	0.200	10.0	30.0	300	Carcinoma
60	29-2368	100	0.032	0.067	5.3	6.1	110	Hodgkin's
61	29-2636	100	0.041	0.054	4.9	1.6	32	Hodgkin's
62	29-2651	100	0.046	0.065	6.5	3.3	41	Hodgkin's
63	29-J-1	100	0.014	0.024	1.9	2.2	41	Osteogenic sarcoma
64	29-J-2	100	0.010	0.015	0.8	2.1	50	Sarcoma
65	29-2866	100	0.030	0.060	2.3	10.4	100	Fibrosarcoma
66	29-2965	100	0.024	0.056	3.3	7.4	133	Melanotic sarcoma
67	30-396	100	0.018	0.060	3.9	7.2	232	Myxofibrosarcoma
68	30-600	100	0.062	0.120	10.4	9.2	94	Lymphoma
69	30-682	100	0.011	0.027	1.2	4.3	60	Fibrosarcoma
70	30-768	100	0.061	0.108	12.6	30.0	77	Lymphoma
71	29-664	50	0.012	0.046	9.6	−7.7	28	Melanotic sarcoma



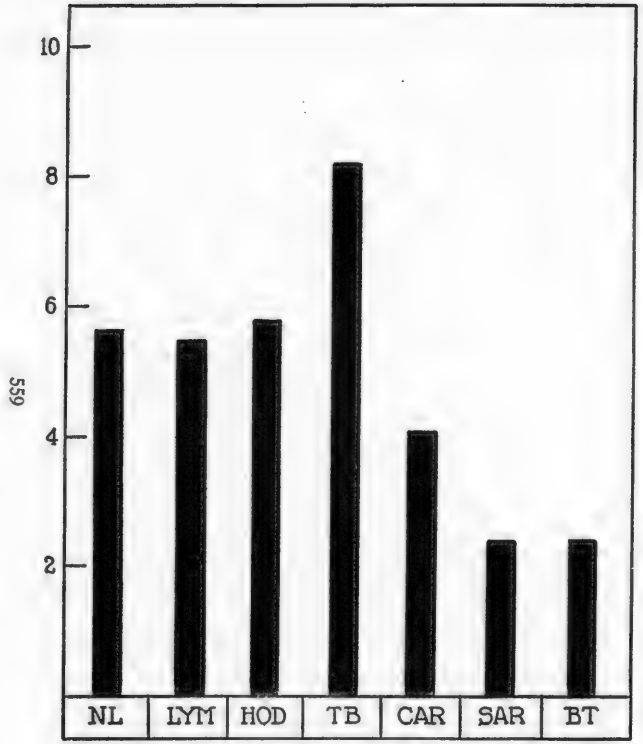
Aerobic glycolysis mg. per mg. hour

CHART 1



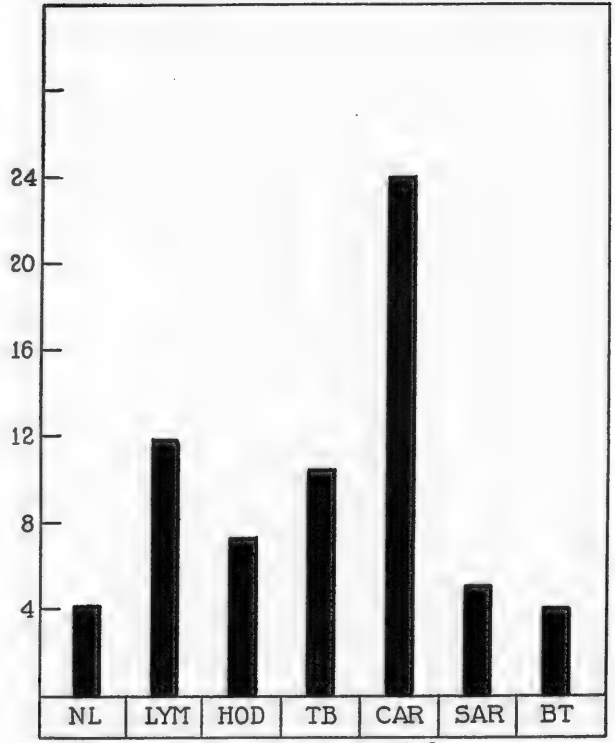
Anaerobic glycolysis mg. per mg. hour

CHART 2



Oxygen consumption cmm. per mg. hour

CHART 3



Warburg's value "U"

CHART 4

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SEROLOGICAL REACTIONS IN PNEUMONIA WITH A NON-PROTEIN SOMATIC FRACTION OF PNEUMOCOCCUS*

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(Received for publication, June 26, 1930)

It has been shown (1) that pneumococci contain two constituents which are chemically and antigenically distinct. One of these, the type-specific component, is a complex polysaccharide, predominantly present in the capsule of the organism; the other, a substance common to the pneumococcus species, is the so-called nucleoprotein, contained for the most part in the body of the cell. That these two chemically distinct fractions are responsible for the production of two qualitatively different antibodies has been demonstrated (1, 2).

The present report is based upon observations made with a third fraction derived from pneumococci and chemically distinct from both type-specific capsular polysaccharide and non-type-specific somatic nucleoprotein. For purposes of reference this substance is designated Fraction C. The chemical nature of Fraction C and the method of purification together with certain experimental observations are presented in a separate communication (3). In this report it is sufficient to state that Fraction C is a non-protein material of somatic origin and appears to be a carbohydrate common to the pneumococcus species. Although final proof of its exact nature rests upon chemical analysis, nevertheless convincing evidence of the separate identity of Fraction C is brought out by the serological reactions to be described.

Material and Methods

Preparation of Fraction C.—The material employed in the serological tests was derived from a degraded, non-type-specific R strain of Pneumococcus. A strain of this character was employed in order to minimize the presence of type-specific carbohydrate. Fraction C was obtained in the following manner: The organisms

* Presented before the American Society for Clinical Investigation at a meeting held in Atlantic City, May 5, 1930.

contained in several liters of full-grown broth culture of an R strain were centrifuged and resuspended in normal salt solution in 60 to 100-fold concentration. The bacteria were then frozen and thawed several times until dissolution had been effected. 0.3 cc. to 0.5 cc. of normal acetic acid was added and the solution boiled for 8 to 10 minutes. The heavy coagulum thus formed was removed by centrifugation. Acidulation and boiling were repeated to insure removal of all acid and heat precipitable material. The final water-clear supernatant fluid, neutralized with normal NaOH, contained Fraction C. Material prepared according to this simple procedure was comparable in reactivity and specificity to more highly purified preparations. Consequently, further steps in purification were not, as a rule, carried out. Some lots, however, were treated by repeated precipitation with 4 to 5 volumes of 95 per cent alcohol.

In the charts the reaction is represented as occurring in dilutions of C substance as high as 1 to 640,000. This figure is only approximate since a quantitative estimation was not made on all lots of material. However, accurate measurement was made with one sample; using it as a standard some of the sera were re-tested and found to react with a 1 to 1,000,000 dilution. Consequently the approximate figures given are believed to be conservative estimates.

The chemical fractionation of numerous bacteria by procedures similar in many respects to that just described, has been reported by others. Pick (4) derived from typhoid bacilli an apparently non-protein substance which reacted especially in immune serum. Zinsser (5) and Zinsser and Parker (6) isolated so-called residue antigens from tubercle bacilli, pneumococci, staphylococci, influenza bacilli, and typhoid bacilli. Laidlaw and Dudley (7) obtained a carbohydrate from tubercle bacilli. Furth and Landsteiner (8) obtained from typhoid and paratyphoid B cultures three fractions, one of which appeared to be non-protein. Day (9) derived from staphylococci an "antigenic specific substance." Lancefield (10) separated a somatic carbohydrate from hemolytic streptococci which she designated Fraction C and which is comparable in many respects to the pneumococcus Fraction C.

Pneumococcus Type-Specific Polysaccharides.—The preparations employed were made according to the method described by Heidelberger and Avery (11).

Pneumococcus Nucleoprotein.—The material was made by precipitation with acetic acid in the cold as described by Avery and Morgan (12).

Precipitin Tests.—0.2 cc. test serum was diluted to 0.5 cc. with physiological salt solution; to this was added an equal volume of varying dilutions of precipitinogens. Final readings were made after the mixtures had been kept in water bath at 37° for 2 hours and over night in the ice box.

Agglutination Tests.—0.5 cc. of varying dilutions of serum was added to 0.5 cc. of heat-killed pneumococci suspended in physiological salt solution. The tubes were incubated at 37° for 2 hours and allowed to stand overnight in the ice box before final reading was made.

Sera obtained at frequent intervals from patients acutely ill with pneumonia or convalescent from the disease have been mixed with varying dilutions of Fraction C and the presence or absence of precipitation noted. Charts 1 and 2 illustrate results which have been repeatedly obtained.

Chart 1 gives the results of observations in a case of Type II pneumococcus pneumonia. Serum obtained on admission to the hospital in the third day of disease reacted with high dilutions of Fraction C.

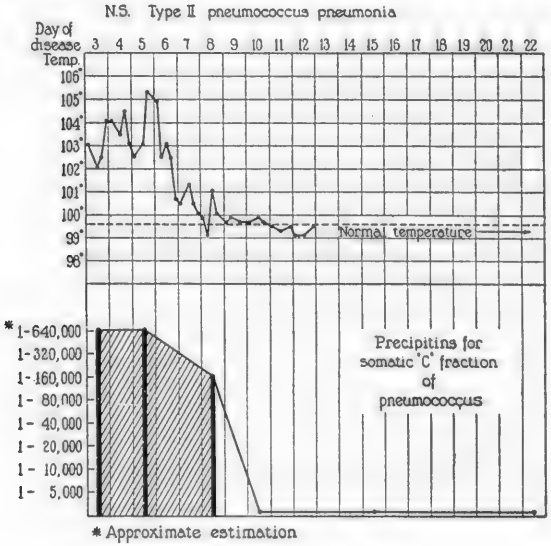

CHART 1

This reactive capacity dropped slightly on the day of crisis and completely disappeared in the next 2 days.

Chart 2 presents a similar course of events obtained with serum derived from a patient suffering from Group IV pneumococcus pneumonia. The phenomenon exhibited in Charts 1 and 2 has been observed in each of 50 patients ill with pneumococcus pneumonia. In each instance it has been found that serum obtained during the acute stage possesses a high titre of precipitins for Fraction C. A day or two after recovery this precipitating power is no longer detectable. The occurrence of the phenomenon is unrelated to the type of Pneu-

mococcus causing infection. The fact that serum obtained from patients on admission possessed, in every instance, anti-C precipitins is evidence of the early appearance of the reactivity. The serum obtained from one patient as early as 18 hours after the initial chill reacted with high dilutions of C substance. Individuals who succumbed to the disease maintained C precipitins until death. The age of patients, whose sera have been tested, ranged from 7 years to

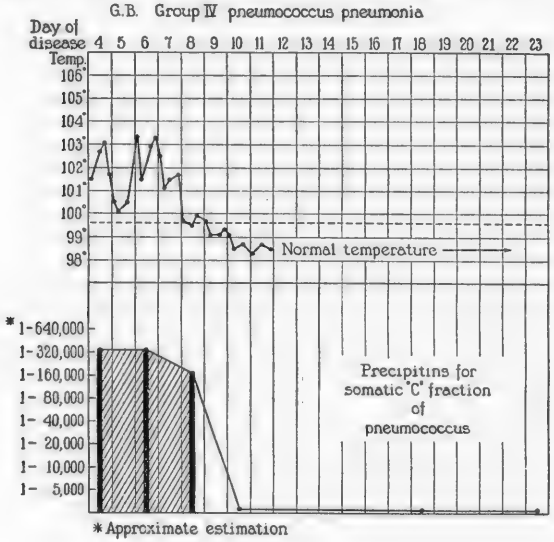

CHART 2

65 years; the phenomenon was present in the youngest and oldest. Sera from normal individuals have not been found to possess demonstrable precipitins for Fraction C.

The observations on the serological reactivity of pneumococcus Fraction C presented in this report are limited to precipitin tests made with sera from patients. However, it may be mentioned that antipneumococcus sera derived from animals, in some instances, precipitate Fraction C. This is particularly demonstrable with the antipneumococcus horse sera[1] used for typing of pneumococci. The

[1] The antipneumococcus horse sera were obtained through the courtesy of Dr. A. B. Wadsworth from the New York State Board of Health Laboratories, Albany, New York.

available lots of antisera of Types I, II and III all precipitate Fraction C, Type III serum apparently reacting in highest titre. Of sera derived from 24 rabbits immunized with either Types I, II, III, or R pneumococci, only 3 possessed demonstrable anti-C precipitins; each of these animals had received heat-killed

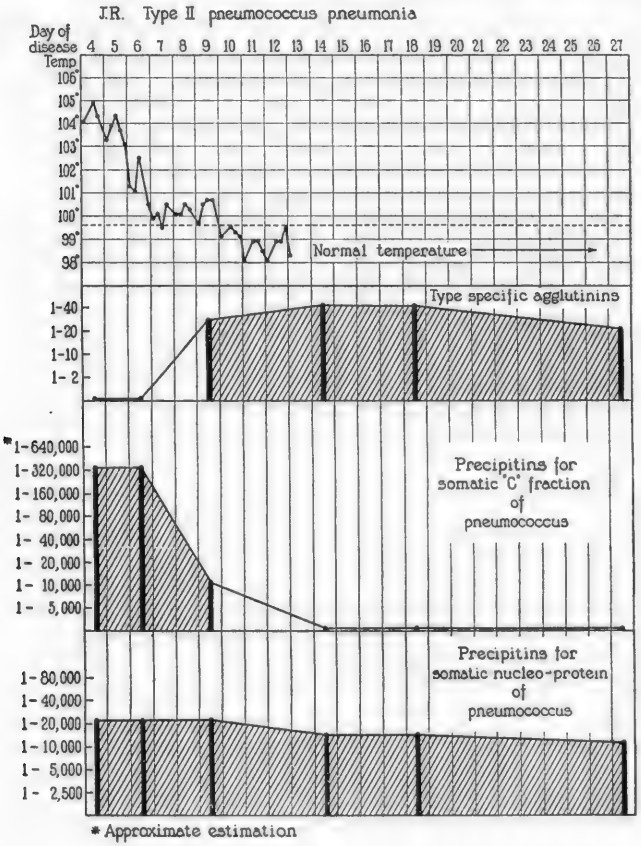

CHART 3

cultures of Type III Pneumococcus. At the present time the conditions which favor the production experimentally of anti-C antibodies are not understood.

For purposes of comparison, sera derived from patients during the course of pneumonia were tested for the presence of type-specific

antibodies and for pneumococcus protein antibodies as well as for C precipitins. The results when charted in relation to the course of pneumonia, present three different curves of antibody titre. Observations of this character are presented in Chart 3.

From Chart 3 it will be seen that type-specific antibodies are absent during the acute illness and are first demonstrable at the time of crisis, whereas precipitins for C substance are present in high titre during the febrile stage and are no longer detectable a few days after

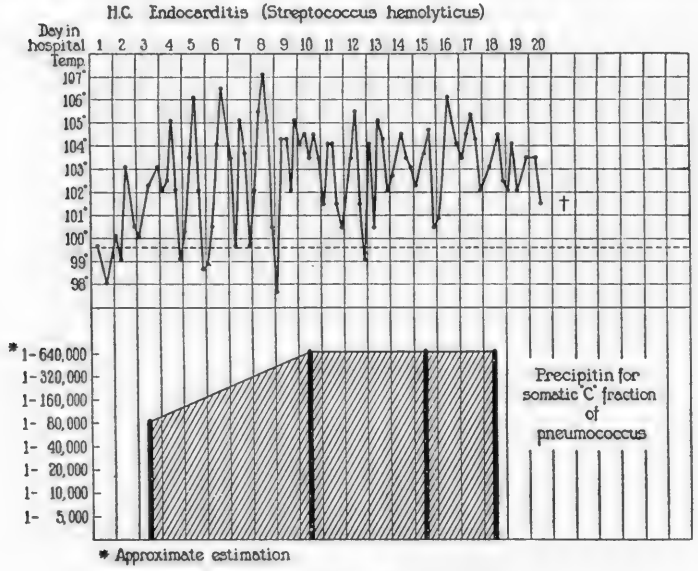

CHART 4

the critical fall in temperature. Antiprotein antibodies do not vary markedly during the course of illness, although there seems to be a slight reduction in titre as convalescence progresses. Observations similar to those given in Chart 3 have been made on twenty cases of pneumonia. It is an interesting fact, therefore, that with three chemically distinct fractions derived from the same bacterial cell, serological reactions involving three qualitatively different antibodies may be demonstrated during the progress of pneumococcus pneumonia.

Up to this point the report has been limited to the results obtained with the sera of patients suffering from pneumococcus infection. A limited number of individuals acutely ill with other febrile diseases have been available for comparative tests. Four cases of hemolytic streptococcus infection ending fatally were observed from admission to the hospital until death. Three of these had streptococcus pneumonia. In each instance their sera precipitated pneumococcus Fraction C equally as well as did sera from cases of pneumococcus pneumonia. The fourth patient had malignant endocarditis; hemolytic

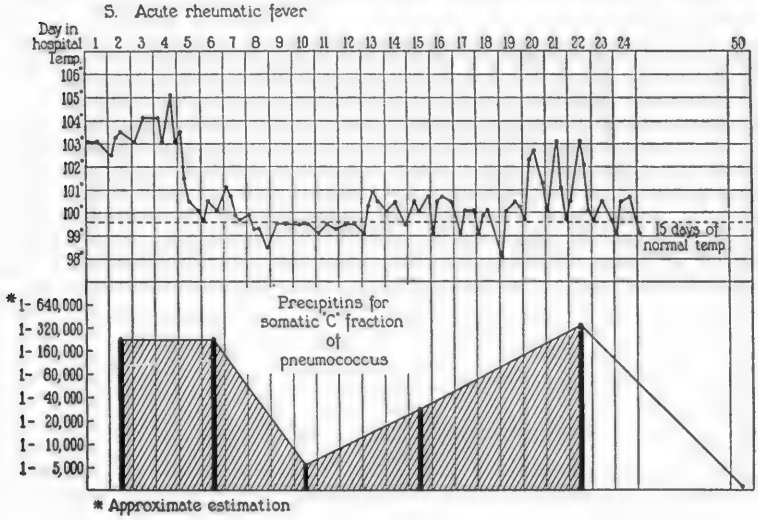

CHART 5

streptococci in large numbers were repeatedly demonstrated in blood cultures. The result of serological tests made with pneumococcus C substance in this case is presented in Chart 4.

As can be seen from Chart 4 the patient's serum maintained throughout the illness the capacity to react in high titre with C substance of Pneumococcus.

Fifteen cases of acute rheumatic fever have been followed by similar serological tests through stages of active disease and remission. Chart 5 records the results obtained in one of this group.

As demonstrated by Chart 5 the course of the temperature and the capacity of the patient's serum to precipitate pneumococcus Fraction C closely parallel each other. The same relations were found to hold in the other fourteen cases of rheumatic fever. All of the patients at the time of elevated temperature exhibited symptoms and signs of active disease.

TABLE I

Reactions with Fraction C in Febrile Diseases Other than Pneumococcus Pneumonia

Patient	Disease	Temperature	*Dilutions of Fraction C		
			1–10,000	1–80,000	1–120,000
Das.	Malaria	104°	−	−	−
Mek.	Lung abscess	102.5°	+	++	+++
Fol.	Cirrhosis of liver	103°	−	−	−
Mit.	Typhoid fever?	104°	−	−	−
Rid.	Pericarditis, rheumatic origin	103°	+	++	++
Reg.	Unexplained anaemia	104°	−	−	−
Hag.	Tuberculosis of lungs	102.5°	±	−	−
Mer.	Unexplained fever—Tbc.?	103.5°	−	−	−
Hin.	Osteomyelitis (staphylococcus)	105°	+++	+++	++
McD.	Measles	102.8°	−	−	−
Pe.	Chicken pox	103°	−	−	−
Mac.	Endocarditis (*Streptococcus viridans*)	104°	+	+	±

* Approximate estimation.

In addition to the hemolytic streptococcus infections and cases of acute rheumatic fever just mentioned, single tests were made on sera obtained in a miscellaneous group of diseases. The results are listed in Table I.

From Table I it can be seen that patients, whose sera precipitated pneumococcus Fraction C, were in each instance suffering from disease associated with Gram positive cocci. The number of cases so far available is too few to attach undue significance to this point. However, the results suggest that etiological agent and serological reactivity with Fraction C may have some causal relationship.

DISCUSSION

The observations recorded in this communication have been made with a constituent of pneumococcus cells which is chemically distinct from both the type-specific capsular carbohydrate and the somatic nucleoprotein. Although pneumococcus Fraction C in all probability is a nitrogenous sugar, it differs from the soluble specific substance in that it possesses no type-specificity and is contained, as a common species constituent, within the body of the cells. Pneumococcus Fraction C appears to be analogous to the somatic carbohydrate isolated from hemolytic streptococci by Lancefield (10).

By testing sera obtained from cases of lobar pneumonia during the progress of the disease two interesting observations were made. The first of these is the early appearance of precipitins for C substance and the second is the complete disappearance of the phenomenon 1 to 3 days after crisis. Sera obtained from patients on the day of admission to the hospital have in every instance reacted with Fraction C. This was true even of cases admitted in the first 24 hours of disease. Since the serum of no normal individual has exhibited this reaction, the appearance of C precipitins is associated closely with the onset of pneumonia. Although an explanation of the phenomenon is not yet available, a possible interpretation is suggested by the so-called "anamnestic" reaction. Cole (13) found that in an animal which had previously been immunized and in which the antibodies in the blood had disappeared a subsequent injection of the original antigen caused antibodies to reappear more rapidly than did the primary injections. Bieling (14) employing two antigens (*B. dysenteriae* and *B. typhosus*), found that the reinjection of one brought back heterologous as well as homologous antibodies. In the course of similar experiments McKenzie and Frühbauer (15) using four antigens of remote biological origin were able to cause heterologous antibodies promptly to reappear by the injection of one antigen. Applying the principles involved in the "anamnestic" reaction to the observations recorded in this paper, it is possible that the occurrence of pneumococcus C precipitins early in pneumonia may be determined by a previous experience of the individual with some suitable bacterial antigen.

In addition to the observations with Fraction C, the investigation

was extended to include a more complete study of the occurrence during pneumonia of antibodies reactive with pneumococcus constituents. In these latter experiments, therefore, tests were made for antibodies reactive with: 1. Type-specific capsular polysaccharide; 2. Non-type-specific somatic polysaccharide; 3. Non-type-specific somatic nucleoprotein. By charting the result of these serological reactions with reference to the course of the disease, the occurrence of each of the qualitatively different antibodies was shown to follow a different course. It is an interesting fact, therefore, that during a *single* infection, the body responds in such a manner that antibodies reactive with different components of the causative organism become demonstrable at different stages of the disease. The results serve to illustrate the complexity of the immunological mechanism which presides over the response to bacterial infection. The observations recorded in this paper also demonstrate that the reactivity of a patient's serum with pneumococcus C substance is not specific for pneumococcus infection. Sera from a limited number of febrile diseases due to other causes have been tested. In this small group, precipitation of C fraction occurred most definitely in those instances where Gram positive cocci were proven to be or were suspected of being the etiological agent. If the implications contained in these few cases are substantiated by a larger number of tests the results suggest some, as yet undetermined, broad relationship existing among certain bacterial infections.

SUMMARY

1. Sera from individuals acutely ill with lobar pneumonia possess the capacity to precipitate in high titre a non-protein somatic fraction derived from pneumococci (Fraction C). Following crisis the reaction is no longer demonstrable.

2. Sera obtained from cases of pneumococcus pneumonia during illness and convalescence have been tested for antibodies specifically reactive with three chemically distinct constituents of Pneumococcus. The results, when correlated with the course of disease, demonstrate differences in the occurrence of each qualitatively distinct antibody.

3. The precipitation of pneumococcus Fraction C is not limited to the sera of individuals ill with pneumococcus infection. But in the

few other cases available for comparative tests, definite reactions have been obtained only in streptococcus and staphylococcus infections and in acute rheumatic fever.

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CUTANEOUS REACTIONS IN PNEUMONIA. THE DEVELOPMENT OF ANTIBODIES FOLLOWING THE INTRADERMAL INJECTION OF TYPE-SPECIFIC POLYSACCHARIDE

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(Received for publication, June 26, 1930)

In a previous communication (1) the occurrence of cutaneous reactions to the polysaccharides and proteins of Pneumococcus during lobar pneumonia was reported. The investigation disclosed the interesting fact that the capsular carbohydrates of Pneumococcus were capable of inciting a reaction when injected intradermally. The character of the response and the conditions under which it was obtained are briefly summarized as follows: The characteristic reaction is the development of an *immediate wheal and erythema* at the site of injection; a positive reaction was first elicited at or about the time of crisis; it was produced only by the polysaccharide homologous in type to that of the Pneumococcus causing the disease; at the time of reaction type-specific antibodies for the homologous organism were demonstrable in the patient's serum.

In contrast to the polysaccharides, the so-called nucleoprotein fraction of the Pneumococcus evoked a *delayed* response which reaches its height in 24 hours. It simulated in appearance the tuberculin reaction. The protein reaction generally appeared first early in convalescence and tended to become progressively more marked during the course of recovery. Circulating antibodies for the nucleoprotein were present in approximately equal concentrations during the periods of acute illness and recovery. The protein reaction bore no specific relation to the type of Pneumococcus producing the infection.

The present report includes further observations on cutaneous reactions to pneumococcus fractions and deals especially with the appearance of antibodies for more than one specific type of Pneumococcus

in the serum of convalescent patients. The individuals whose sera presented these unusual reactions had received repeated intradermal injections of the pneumococcus carbohydrates. The possible relationships between the repeated injection of the specific substances and the antibody formation will be discussed.

Materials and Methods

A. Skin Test Materials

1. Type-Specific Capsular Polysaccharides.—The type-specific polysaccharides were obtained in purified form from Types I, II and III Pneumococcus by the method of Heidelberger and Avery (2). They are chemically and serologically distinct and have been identified with the capsular material of the cell. The Type II and Type III polysaccharides are nitrogen-free; that of Type I contains 5 per cent nitrogen as part of the sugar molecule (3). The polysaccharides were diluted in physiological salt solution to a concentration of 1:10,000 and 0.1 cc. containing 0.01 mg. of the specific soluble substance was injected intracutaneously. Skin tests were repeated at intervals throughout the disease and convalescence.

2. Nucleoprotein.—The nucleoprotein was prepared by the method of Avery and Morgan (4) from an R strain derived from a Type II S organism. The material was standardized on the basis of nitrogen content and 0.01 mg. of protein in 0.1 cc. of physiological saline was used in all tests. These tests were repeated at approximately weekly intervals during the patient's stay in hospital.

B. Titration of Sera for Antibodies

At intervals during the acute illness and convalescence samples of serum were obtained and tested for the presence or absence of circulating antibodies.

1. Type-Specific Antibodies.—The presence in the serum of type-specific antibodies was determined by (a) agglutination, and (b) passive protection of mice.

a. Type-Specific Agglutinins.—0.5 cc. of varying dilutions of the serum to be tested was mixed with 0.5 cc. of a heat-killed suspension of type-specific pneumococci. Final readings were made after the mixtures had been incubated for 2 hours in the water bath and then placed in the ice box overnight.

b. Passive Protection of Mice.—The capacity of patient's serum to protect mice against infection was tested by the usual technique. 0.2 cc. of serum diluted to 0.5 cc. with salt solution was employed. So far as possible each serum was tested against pneumococci of Types I, II and III. The cultures used were invariably fatal for mice in doses of 0.0000001 cc.

2. Antibodies for Nucleoprotein.—Antibodies for the nucleoprotein material were determined by the precipitin reaction as previously described (1).

A study of the cutaneous response to the injections of the type-specific polysaccharides of the Pneumococcus and of the occurrence of circulating antibodies has been made in 74 cases of lobar pneumonia. The results confirm earlier observations and may be briefly summarized as follows: Positive reactions were elicited only with the homologous polysaccharide at crisis or shortly thereafter; homologous type-specific antibodies were demonstrable in the serum of each reactive patient. In no instance was a positive skin reaction to a polysaccharide obtained in the absence of circulating antibodies for the type of Pneumococcus from which the polysaccharide was derived. Table

TABLE I

The Incidence of Cutaneous Reactions to the Homologous Polysaccharide in Convalescent Patients

Type of infecting organism	No. patients	No. positive reaction	Per cent giving positive reactions
I	21	21	100
II	17	10	58.8
III	9	4	44.4

13 recovered cases of Group IV—no reactions to polysaccharides of Types I, II, III.

5 recovered cases of Type II (atypical)—no reactions to polysaccharides of Types I, II, III.

9 fatal cases of various types—no reactions to polysaccharides of Types I, II, III.

I shows the relative frequency of positive reactions to the homologous type of polysaccharide in patients convalescent from Type I, II or III pneumonia. All but 4 of the Type I cases were treated with anti-pneumococcus Type I serum.

From Table I it can be seen that positive cutaneous reactions were obtained in all of the Type I cases and in approximately 50 per cent of the Types II and III patients. The polysaccharides of Types I, II and III elicited no response in the 13 Group IV and the 5 atypical Type II patients. The absence of reactivity in the 9 fatal cases agrees with earlier experiences in that in patients with lethal termination no reaction was obtained. 12 patients convalescent from Type II or Type III pneumonia also failed to react but in 8 of them no reaction

would be expected since their sera contained no circulating antibodies; the sera of the remaining 4 patients contained circulating antibodies but no reaction to the polysaccharide occurred. Thus, of the entire group of patients included in Table I a positive skin reaction was expected in 39 by virtue of the fact that they were recovered cases of Type I, II or III infection whose sera contained type-specific antibodies. Positive reactions were obtained in 35 of them.

The cutaneous response to the nucleoprotein fraction of the Pneumococcus was studied in the same group of cases. The results have confirmed the validity of the earlier conclusions. The delayed protein reaction appeared first during convalescence. The reactivity of the patient did not appear to be influenced by the titer of circulating antibodies for the protein fraction.

Occurrence of Antibodies to Heterologous Types of Pneumococcus

Other observers have repeatedly demonstrated the occurrence of circulating antibodies for the homologous type of Pneumococcus at or about the time of crisis. The present investigation confirms these results and, in addition, discloses the fact that at the time when type-specific antibodies are first demonstrable in the blood the patient becomes reactive to the cutaneous injection of the homologous type-specific polysaccharide. The reactions have been specifically limited to the homologous types. In the present study repeated tests were made with the type-specific polysaccharides of Types I, II and III with a view to determining the length of time through which the reactive capacity might persist. In view of the fact that positive results at the time of recovery were strictly limited to the homologous type, it was rather surprising to observe after repeated tests that a positive skin reaction was obtained in certain instances with a polysaccharide of heterologous type as well. With the appearance of the skin reaction to the heterologous polysaccharide, circulating antibodies for the heterologous type of Pneumococcus were demonstrable in the patient's serum, in addition to those for the homologous type. The chief interest in this report, therefore, is concerned with the development of specific antibodies for more than one type of Pneumococcus in patients who have repeatedly received intradermal injections of type-specific carbohydrates. These "additional" antibodies were

characterized by the fact that they were heterologous in type to that of the infecting organism and were not demonstrable at crisis but appeared 1 to 3 weeks after the patient's recovery. These features were disclosed as a result of what at first appeared to be a discrepancy in specificity of the cutaneous reaction. The results can be more clearly brought out by describing in detail the course of events in one patient.

M. A., a girl of 14 years, suffering from Type II pneumonia was admitted on the second day of disease. No circulating antibodies were demonstrable in the patient's serum at this time. Crisis occurred on the tenth day. On the thirteenth day skin tests with the polysaccharides of Types I, II and III revealed a positive reaction only to the Type II material. The serum taken shortly thereafter contained circulating antibodies for Type II Pneumococcus alone. 8 days after the first test the skin tests were repeated and at this time positive reactions occurred both to Type II and Type I polysaccharides. At this time her serum contained antibodies for Type I as well as for Type II Pneumococcus. (See Chart 1.)

Because of the appearance of heterologous antibodies and skin reactions a series of patients was studied in order to determine the frequency with which the phenomenon occurred. Skin tests and antibody determinations were made at varying intervals in 18 patients. Of these, 3 were cases of Type I pneumonia, 7 of Type II, 3 of Type III and 5 of Group IV. At or about the time of crisis antibodies for the homologous type of Pneumococcus appeared in all but 2 of the Types I, II and III patients. In the Group IV cases tests were not carried out with the homologous organism. However, in no instance were heterologous antibodies demonstrable at that time. As convalescence progressed, repetition of skin tests and antibody determinations revealed that there had *developed*, since they were previously absent, specific antibodies and a positive skin reaction for at least one of the heterologous types of Pneumococcus. Table II shows the intervals between the appearance of homologous and heterologous antibodies in relation to the day of disease and recovery.

From Table II it can be seen that in 10 of the 18 patients heterologous antibodies were detected during convalescence; in 3 Type I patients, 4 Type II, 2 Type III, and 1 of Group IV. In the great majority of cases the presence of circulating antibodies for the heterologous type was first indicated by a positive skin test to the polysac-

TABLE II

Differences in Time of Appearance of Homologous and Heterologous Antibodies in Patients Receiving Skin Tests with Type-Specific Polysaccharides

Patient	Antibodies to homologous type		Antibodies to heterologous types				Day of crisis
	Type	Day observed	Type			Day observed	
L. H. (serum), No. 7165	I	3			III	17	6
M. P. (serum), No. 7263	I	8		II		14	5
F. R., No. 7253	I	9		II		22	7
M. C., No. 7183	II	14	I			19	5
G. M., No. 7248	II	10	I			19	9
E. C., No. 7269	II	11	I			18	9
M. A., No. 7261	II	15	I			22	10
H. R., No. 7262	III	6		II		19	
			I			26	8
K. R., No. 7143	III	5	I	II		21	4
A. P., No. 7211	IV	Not determined	I			26	
					III	44	7
M. C., No. 7189	II	15	0	0	0		8
I. S., No. 7195	II	9	0	0	0		6
C. Q., No. 7225	II	0	0	0	0		7
J. F., No. 7171	III	0	0	0	0		10
G. B., No. 7178							8
R. F., No. 7256	IV	Not determined	0	0	0		9
T. R., No. 7197							5
G. R., No. 7236							8

charide of that type. The type of Pneumococcus for which heterologous antibodies developed was in 7 instances Type I, in 4 Type II, and in 2 Type III. In 3 cases specific antibodies for 2 heterologous types appeared. Although the homologous antibodies were detectable at the time of crisis, those for heterologous types were usually noted in the second week of convalescence, the earliest being 9 days after recovery, the latest 37 days after recovery. The period of time during which antibodies were detectable in the serum and skin reactivity remained positive varied from a few days to several months.

These observations led to the hypothesis that the mechanism involved in the production of antibodies for heterologous types of Pneumococcus was associated with the intradermal inoculations of the type-specific

TABLE III

The Incidence of Type-Specific Antibodies in the Control Series of Patients

Type of infecting organism	No. cases	Antibodies for homologous type of Pneumococcus	Antibodies for heterologous types of Pneumococcus
I	6	5	1*
II	6	6	1*
III	2	2	0
Group IV	7	Not determined	1**

* Type III antibodies present during disease and recovery.

** Type II antibodies present during disease and recovery.

polysaccharides. In order to obtain more information on this point the next 21 pneumonia patients admitted to hospital were studied as controls. In this series no injections of the specific polysaccharides were made but the serum was tested at intervals for circulating type-specific antibodies to Pneumococcus Types I, II and III. In the Group IV cases tests were not made for antibodies reactive with the infecting organism. In most of the 21 cases homologous antibodies were observed at the time of recovery. However, in striking contrast to the group of patients which had received repeated injections of the specific soluble substance, no patient in the control series *developed* antibodies for a heterologous type. In only 3 patients (1 each of Types I, II and Group IV) antibodies for a type other than that of the infectious agent were observed. In these three instances the presence

of the heterologous antibodies was detected in low titre early in the acute illness and the concentration of the antibodies did not increase during convalescence. They, therefore, bear no apparent relation to the present infection but are presumably attributable to a preexisting state. This interpretation is substantiated by the fact that some normal individuals possess in their serum type-specific antibodies.

Table III presents the distribution of the types of Pneumococcus in the control group of patients and the number in whom circulating antibodies were detectable.

The 21 patients who served as controls were comparable to the test group with regard to age incidence, distribution of types of infecting Pneumococcus, severity of the infection and period of observation.

The patients in whom heterologous antibodies were observed, received injections of 0.01 mg. of the type-specific capsular polysaccharides derived from the Pneumococcus of Types I, II and III. The average number of injections before the appearance of antibodies for the corresponding type of Pneumococcus was three. In 3 cases the antibodies were detected after a single injection. The largest total amount which was followed by heterologous antibody production was 0.05 mg. whereas in other cases heterologous antibodies did not develop even after the injection of as much as 0.08 mg. of the polysaccharides. These irregularities demonstrate the fact that the production of antibodies is not solely dependent upon the quantity of polysaccharides injected. The average time between the last injection and the appearance of the antibodies was 6 to 10 days but in one case they were first detected 20 days after the last injection. The exact data regarding the individual patients in whom antibodies for heterologous types of Pneumococcus developed are presented in graphic form in composite charts. These charts show in relation to the day of disease, the time of appearance of type-specific agglutinins and mouse protective antibodies for homologous and heterologous types of Pneumococcus, the time, number and results of skin tests with the 3 type-specific polysaccharides and the period during which the observations were continued.

DISCUSSION

The primary interest of the present work centers in a group of 18 cases of lobar pneumonia 10 of whom, in convalescence, developed

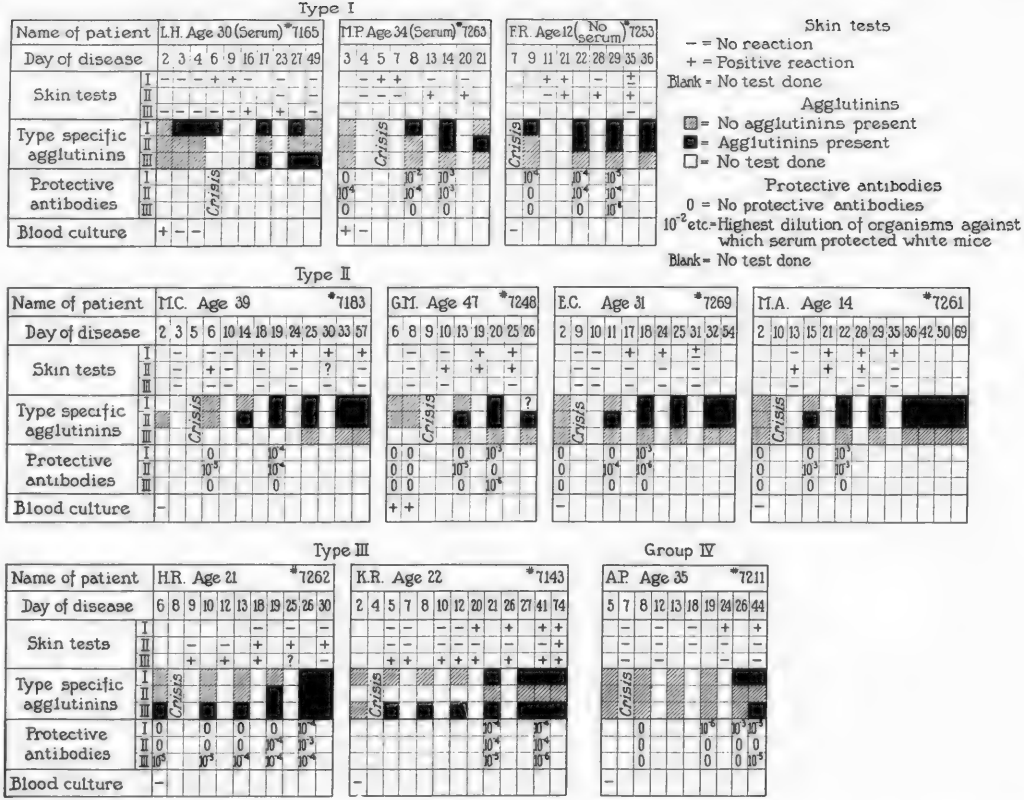

CHART 1. The development of heterologous antibodies in patients receiving intradermal injections of type-specific polysaccharides.

circulating antibodies for at least one type of Pneumococcus heterologous to that etiologically related to the disease. All of the patients in whom the phenomenon was observed had received repeated intradermal injections of 0.01 mg. of specific capsular polysaccharide from each of Types I, II and III Pneumococcus. Antibodies for the homologous type of Pneumococcus appeared at about the time of crisis. Antibodies for heterologous types, on the other hand, appeared usually in the second week of convalescence after 1 to 5 injections of the respective polysaccharides.

A control group of patients, 21 in number, received no skin tests. Their sera, in the great majority of instances, contained antibodies for the homologous type of Pneumococcus. In 3 of these cases antibodies for a heterologous type were present but the time of appearance differed from that of the antibodies observed in the first group in that they were detectable in low concentration not only in convalescence but during the acute illness as well. Nor was any increase in concentration observed during convalescence. Antibodies to the heterologous types which were encountered in these 3 cases, apparently preexisted and their appearance is presumably unrelated to any incident occurring in the course of the present infection. The validity of this supposition is borne out by the fact that type-specific antibodies may be found in the serum of certain normal individuals, who give no history of lobar pneumonia. But, in no case were heterologous antibodies first detected in convalescence unless the patient had previously received intradermal injections of the type-specific polysaccharides.

Many investigators have noted the presence of specific antibodies for the homologous strain or type of Pneumococcus in the blood of pneumonia patients at the time of crisis. Bacteriotropins (5, 6, 7), agglutinins (5, 7, 8, 9, 10, 11, 12) and protective antibodies (13, 14, 15, 6, 12) have all been demonstrated but so far as can be discovered, in no instance has the development of antibodies for a heterologous type been described. Chickering (11) in 1914 followed the course of agglutinin production to all types throughout convalescence in 40 cases but found only antibodies for the homologous type. Clough (7) in 1919, using the serum of patients convalescent from pneumonia due to known types of Pneumococcus, obtained similar results with phagocytic and agglutination reactions. In the sera of 25 normal

individuals, however, Clough (16) found protective antibodies for Type I in 4 of 18 tests, for Type II in 8 of 18 tests; and of 19 tests for Type III antibodies, 8 were positive. Ward (20) tested the capacity of the defibrinated blood of normal individuals to inhibit the growth of pneumococci of Types I, II and III. In each instance a variable degree of pneumococcidal action was observed against one or more types of the organism. Furthermore, this property was present in the blood of patients early in the course of pneumonia even against the type of Pneumococcus producing the infection. Robertson and Cornwell (21), employing serum-leucocyte mixtures from normal human beings, found that the blood of all persons tested by this method possessed pneumococcidal properties for at least one type of Pneumococcus and in the majority of instances for two or more types.

In evaluating the factors involved in the production of antibodies for types of Pneumococcus heterologous to that causing the infection several possible mechanisms may be considered.

It is possible that antibodies for other types were present before the onset of disease and were masked during the acute infection only to return with convalescence. Or that, present in minimal amounts before the disease, the infection served to cause an outpouring of antibodies in detectable concentrations,—the anamnestic reaction first observed by Cole (17).

Interconvertibility of types as described by Griffith (18) and later studied by Dawson (19) offers a possible explanation. One might assume that with the development of type-specific antibodies to the homologous organism a change of type occurred and, as a result, antibodies to the secondary type appeared.

The evidence presented in this report implicates the polysaccharides employed in intracutaneous tests and suggests that they were antigenic. On the basis of this assumption, the conditions under which the investigation was carried out may have favored antibody production. No previous data are available regarding the antigenicity in humans of the purified type-specific polysaccharides of the Pneumococcus. On the basis of animal experiments in this laboratory these substances, in the pure state, are considered non-antigenic. It is possible, however, that in the process of recovery from infection, a highly reactive state exists in the human organism which responds to stimuli otherwise

ineffective. In the removal of the inflammatory material from its focus in the lungs, as takes place in pneumonic resolution, the absorbed material may act as an adjunct to the polysaccharides (the "Schleppe" of the German investigators) forming a combination possessing antigenic properties. The polysaccharides which, under ordinary conditions, are haptens, may then acquire antigenic properties and determine the specificity of the antibodies produced. It is of course also possible that the particular preparations used in this study, although they were prepared with great care, still contained minute amounts of combined polysaccharides.

At present it is impossible to offer any final statement as to the exact mechanism by which antibodies for the heterologous types are produced. Further work is being carried on with normal individuals and patients in an attempt more completely to elucidate the factors which play a rôle in the development of these heterologous antibodies.

SUMMARY

The majority of patients convalescent from pneumonia due to Types I, II and III Pneumococcus develop at the time of recovery circulating antibodies for the homologous type of organisms. At the same time an immediate wheal and erythema reaction followed the intradermal injection of the homologous type-specific polysaccharide in 100 per cent of Type I patients, 58.8 per cent of Type II patients, and 44 per cent of Type III patients.

In a group of 18 patients repeatedly tested with the type-specific polysaccharides, 10 developed in the second or third week of convalescence circulating antibodies for one or more heterologous types. In none of 21 control patients was this phenomenon observed.

It is suggested that the development of circulating antibodies for heterologous types of Pneumococcus was associated with the previous intradermal injections of the type-specific polysaccharides.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

VI *a*. Trypanosoma lewisi Infection in Normal Albino Rats
VI *b*. Trypanosoma lewisi Infection in Suprarenalectomized Adult Albino Rats

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(Received for publication, June 25, 1930)

VI a. T. lewisi Infection in Normal Albino Rats

Though much evidence has been accumulating during recent years to indicate the importance of the reticulo-endothelial cells in resistance and antibody formation, little is known of the more fundamental controlling influences of this mechanism. In previous studies (1–5) we have gathered evidence of the importance of the suprarenal gland in antibody formation through the influence of the water balance in the tissues of the body. That the suprarenal glands are essential in the mechanism of natural resistance has been established through the work of several investigators and in previous work in this laboratory (6–9). In an effort to determine what part the suprarenal gland and other glands of internal secretion play in resistance to protozoan diseases, *T. lewisi* infection, a common disease of rodents, was studied.

T. lewisi infection in the albino rat offers certain advantages for experimental study.* Spontaneous infection is rare, but the disease can be readily transmitted by inoculation of infected blood. Its course can be quantitatively studied. The severity of the infection can be estimated by counts of the trypanosomes in the peripheral blood stream and by estimation of the coefficient of variation in the size of the parasites during the reproductive phase of the infection (12). They produce a mild disease in adult rats and recovery occurs within 4 to 5 weeks, with an acquired immunity that remains permanent.

* For a review of the literature on *T. lewisi* infection, see Laveran and Mesnil (10) and Taliaferro (11).

The wide variation in the course and duration of the infection reported by previous workers (11, 13, 14) may be in part due to the fact that the factors of stock, age, weight of the rats, diet and the environmental conditions of the animals were not uniform. We have kept these factors as constant as possible.

The course of *T. lewisi* infection was studied in 40 normal adult albino rats and was made the basis of comparison with infections in suprarenalectomized, nephrectomized, splenectomized, gonadectomized, and thymectomized rats.

Method

The rats were all of Wistar Institute stock, raised in our laboratory. Except where otherwise specifically stated, they were 3 months of age, and ranged in weight from 150 to 225 gm. They were kept under constant environmental conditions and maintained on a standard adequate diet.

The strain of the *Trypanosoma lewisi* was obtained through the courtesy of Dr. Linton of Columbia University and was maintained by weekly transfers. 1 cc. of blood from rats infected with *T. lewisi* was drawn from the heart and was diluted with 9 parts of physiological salt solution containing 1 per cent sodium citrate. 1 cc. of this dilution was injected intraperitoneally into a series of rats. Red blood cell counts were made at frequent intervals. Smears of the peripheral blood were made daily during the first week and then at 2 day intervals thereafter until the trypanosomes disappeared from the blood stream. Trypanosome counts were estimated and the curve of the infection determined in each instance.

The Course of the Experimental Infection in the Normal Adult Albino Rat

Following intraperitoneal injection the trypanosomes multiplied in the peritoneal cavity for a period of 1 to 3 days. Within 24 to 48 hours trypanosomes appeared in the peripheral blood. They rapidly multiplied and swollen forms became numerous. By the fourth day the count exceeded 100,000 per cubic millimeter. Multiplication forms were present during the first 7 to 9 days and from then on only adult forms were seen. The average interval from the onset to the height of the infection was 7.5 days, the range 5 to 18 days. The number of trypanosomes at the height of infection varied from 115,000 to 800,000 per cubic millimeter. The average number was 336,000 per cubic millimeter. The average duration of the infection was 27.4 days. In two instances the duration of the infection was less than 3

weeks, in sixteen 3 to 4 weeks and in twenty-two 4 to 5 weeks. No instance of infection in our normal rats exceeded 35 days. The average duration of the infection was the same in males and females. In most instances the trypanosomes disappeared from the blood gradually, but in six instances the number of trypanosomes dropped within 24 hours from a high count to zero. In general these findings are consistent with those of previous investigators. The duration of the infection in the rat is not as varied if the factors of stock, age, weight, environment, care and diet are kept uniform.

Symptoms of the Infection.—Laveran and Mesnil (10) observed that very young rats failed to gain weight during the first few days of the infection. A definite clinical syndrome in the adult rat was noted when *T. lewisi* infection was first introduced into our stock. During the first 48 hours, the rat was quiet, his appetite was poor, the eyelids were edematous and sticky, and edema of the penis was present. These symptoms rapidly subsided and the rats appeared normal during the rest of the infection. In two instances during the second week of the infection partial paralysis of the hind limbs was observed which disappeared within 3 weeks. After the first two or three transfers of the strain of *T. lewisi*, symptoms of edema of the lids and penis were no longer noted and no further instance of paralysis was observed. The infection was associated with fewer symptoms when the *Trypanosoma lewisi* strain had undergone several passages.

A moderate anemia developed during the first few days of the infection which was most severe at the height of the infection. The red cell count rarely dropped below 4,500,000. The anemia was associated with the appearance of *Bartonella muris* bodies in the red cells. From experimental studies reported in a previous communication (15) it was found that the virus of *Bartonella muris* anemia could be separated from the trypanosome infected blood by passage through young rabbits. The concomitant anemia was more severe in 3 week old rats than in mature rats and the *T. lewisi* infection ran a more prolonged and severe course in the young rats. Death from trypanosome infection in very young rats may occur with a severe anemia, the red cell count dropping to 1,000,000 or less. *Bartonella muris* bodies are present in large numbers in the red cells. Autopsy of the fatal cases reveals icteric tint to the subcutaneous fat, enlargement of the spleen and fatty changes of the liver, heart and kidneys.

Pathology of the Trypanosoma lewisi Infection in the Normal Adult Rat

A series of 19 rats were inoculated with blood from a rat injected with *Trypanosoma lewisi* and killed at intervals of 2 days from the first day of infection to the 38th day after inoculation. Macroscopically the only pathological changes were noted in the spleen. It enlarged progressively until at the height of the infection it was 6 to 7 times the normal size, amounting to 1.55 per cent of the body weight as compared with 0.27 per cent normally. It diminished in weight after the 10th day but at the end of the infection it was still larger than normal. Histological studies show a progressive enlargement of the follicles and marked congestion of the pulp. The reticular and endothelial elements became engorged with red blood cells, were greatly increased in size and many underwent disintegration. Some of the Kupffer cells of the liver show erythrophagocytosis. In the later stages of the infection the follicles of the spleen diminish in size, the congestion disappears and there is some increase in the connective tissue of the pulp. No trypanosomes were found in the tissues of rats killed at the height of the infection when several hundred thousand trypanosomes per cubic millimeter were present in the peripheral blood stream.

In mice infected with pathogenic trypanosomes the spleen may hypertrophy to 60 times the normal size (16). Taliaferro (11) states that splenic enlargement is more marked in the severe trypanosome infections of mouse, rat, guinea pig and dog,—which die with large numbers of parasites in their blood,—than in the goat, sheep, rabbit and cattle in which trypanosomes are only rarely found.

T. lewisi infection in the rat results in severe injury to the reticular and endothelial elements of the spleen. Evidence of such injury is afforded by the concomitant occurrence in many rats of the *Bartonella* anemia at the height of the trypanosome infection. *T. lewisi* infections produce the same effect as splenectomy. Cannon and McClelland (17) found that repeated injections of large amounts of India ink over a long period of time in normal rats is followed by the appearance of *Bartonella* anemia. As much as 60 to 80 cc. of India ink (4 per cent suspension) was used per rat. They attribute this

anemia to physiological "blockade" of the reticulo-endothelial elements.

Complement Fixation in Trypanosoma lewisi Infection

Although complement fixation in diseases due to the pathogenic trypanosomes has been extensively investigated (11), no studies on complement fixation in *T. lewisi* infection in the rat have been reported to our knowledge.

Manteufel (18) used aqueous and alcoholic extracts of organs of rats infected with *T. lewisi* in *T. equiperdum* studies in horses with contradictory results. Besseman and Leynen (19) in studying methods of obtaining sensitive antigens for use in diagnosis in *T. equiperdum* (dourine) used among other things *T. lewisi* as a source of the test antigen and found it unsatisfactory.

Studies on the complement fixing antibodies of rats recovered from *T. lewisi* were made in an effort to obtain a comparative standard for antibody formation under different conditions. Aqueous and alcoholic antigens were used, prepared from the liver and spleen of rats infected with *T. lewisi*. Saline washings of heated and unheated cultures of *T. lewisi* grown on Novy and McNeal medium were also tested.

Methods.—The saline extracts were made as follows: The spleens of infected rats were macerated and ground in a mortar and shaken with 30 cc. of physiological salt solution per spleen during 30 minutes. The mixture was rapidly filtered and the filtrate preserved in 0.5 per cent phenol. Only freshly prepared extracts were used. The livers of infected rats were macerated with saline and the volume was brought up to 15 cc. per gram of liver. The suspension was shaken thoroughly for 30 minutes, left over night in the ice chest, again shaken and filtered. The anticomplementary titer of the antigens was determined and one-fourth the anticomplementary unit was used in each test. The tests were carried out as follows: 0.1 cc. of inactivated serum, 0.1 cc. of antigen in the dilution estimated by previous titration and 0.1 cc. of guinea pig serum diluted as indicated by previous complement titration (2½ units). The total volume was brought up to 1 cc. and incubated during 1 hour at 37°. The hemolytic system, consisting of 0.1 cc. of 5 per cent suspension of washed sheep cells and 0.1 cc. of amboceptor in dilution to equal 2½ units was then added.

We have found that during the course of the infection the serum of the infected rat is anticomplementary in very high dilutions, some-

times higher than 1/1000, owing to the fact that both antigen and antibody are present in the serum of the rat during the infection.

Complement fixation in rats recovered from the infection for several weeks were tested with the antigens described. All the antigens gave positive results with positive sera but the strongest reactions were obtained with saline extracts of spleen and liver and saline washings of trypanosome cultures. A sample protocol is given below.

Sera	Saline extract spleen	Saline extract liver	Saline washings trypanosome cultures
Pooled positive*	1/400	1/160	1/600
Positive Rat 1	0	0	0
Positive Rat 3	1/700	1/80	1/1000
Positive Rat 4	1/100	1/200	1/400
Positive Rat 5	1/200	1/20	1/200
Pooled negative**	1/10	1/10	1/10
Negative Rat 2	0	0	0

* Positive sera were obtained from rats 2 weeks after they had recovered from *T. lewisi* infection.

** Negative sera were obtained from normal uninfected rats.

Further studies are in progress to determine the course of antibody formation during the life of the rat following infection.

Since the original observations of Rabinowitch and Kempner (20) it is known that a rat which has recovered from an infection with *T. lewisi* cannot be reinfected. The mechanism of this permanent immunity is not understood but studies on antibody formation suggest that the immunity is primarily a humoral one.

Laveran and Mesnil had observed the phagocytosis of *T. lewisi* in the peritoneal cavity of immune rats reinjected with trypanosomes. That phagocytosis by monocytes and circulating leucocytes plays a part in the immune reaction is evident, but many observers have failed to note phagocytosis of trypanosomes.

SUMMARY

T. lewisi infection was studied in a large series of normal adult and young albino rats. The importance of using rats of the same stock, age, and weight for comparative studies with this infection is empha-

sized. The infection produced by intraperitoneal inoculation of blood of an infected rat lasts about 1 month. The height of the infection is reached at about the seventh day. *T. lewisi*, though never fatal in the adult rat, produces a definite disease entity with a progressive splenomegaly during the first 10 days of the disease. Histologically there is hyperplasia of the follicles and congestion of the pulp with marked erythrophagocytosis by reticular and endothelial elements. The spleen diminishes in size with the recovery of the animal. A moderate anemia develops, due to the concomitant infection with *Bartonella muris* viris. During the course of the infection the best index of the resistance of the animal is afforded by the daily trypanosome counts, by the interval during which developmental forms are present and by the duration of the infection. The serum of the rat during this period is highly anticomplementary. After recovery complement fixing antibodies can be detected for a long period of time. The best antigens for the detection of complement fixing antibodies are saline extracts of rat spleens removed at the height of *T. lewisi* infection and saline washings of unkilled cultures of trypanosomes.

VI b. T. lewisi Infection in Suprarenalectomized Adult Albino Rats

It has been established that suprarenalectomy in albino rats lowers their resistance to toxins, chemical poisons and bacterial infections and diminishes their capacity for antibody formation (6–9). In an effort to determine the rôle of the suprarenal gland in resistance to protozoan infections, *T. lewisi*, a common protozoan of rats, was studied. In the first part of this communication the course of the infection and its pathology was described. Experiments were next carried out to determine the effect, if any, of suprarenalectomy on the acquired immunity to *T. lewisi* of rats recovered from a first infection.

The experiments were divided into two groups. In the first group eighteen adult rats were suprarenalectomized and in five the suprarenal areas were traumatized. 6 days after the operation all the rats were injected intraperitoneally with 1 cc. of a 10 per cent dilution of whole blood drawn from a rat infected with *T. lewisi*. Smears and counts of the peripheral blood were made at frequent intervals.

The results of this experiment are given in Table I and Fig. 1. It is to be noted that of the 18 rats, 12 or 67 per cent, died within 2 to 19

days. The average duration of life of these rats with a fatal infection was 5.8 days after injection with *T. lewisi*, and the average interval in days between the onset and the height of infection was 4.2 days. The trypanosome count was not greater in the suprarenalectomized rats than in normal rats. Apparently, however, toward the height of the infection, the toxic effect of the trypanosomes was sufficient to kill a large percentage of the suprarenalectomized rats. The average num-

TABLE I

The Effect of Bilateral Suprarenalectomy and Unilateral Nephrectomy on Trypanosoma lewisi Infection

Operation	No. of rats	Days between operation and infect.	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection (Expressed in thousands)				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
Bil. supra. fatal instances	12	6	5.8	2	19	5	4.2	2	8	4	220	10*	640	212	—	—
Bil. supra. recovered instances	6	6	25	12	31	26.5	8.6	5	11	9	338	160	640	305	—	—
Bil. supra. recovered and fatal instances	18	6	12.5	2	31	6	5.9	2	11	5	229	10*	640	212	33	67
Trauma to supra. area	5	6	24	16	28	26	6.4	5	8	6	380	166	700	300	100	0
Unil. nephrect	8	6	26.7	6	45	32	8	6	10	8	330	100	890	290	100	0
Unoperat. adult normals	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0

* Animal died 24 hours after infection.

ber of trypanosomes in the suprarenalectomized rats at the height of infection was 338,000 per cubic millimeter as compared with 337,000 for those which had been normal. The average interval between the onset and the height of the infection in those rats which survived the infection was 8.6 days. The average duration of the infection in the

surviving suprarenalectomized rats was 25 days as compared to 27.4 days of the normal group.

During the same period 20 rats were suprarenalectomized as controls. Of these rats, 80 per cent survived more than 1 month. In experiments with several hundred suprarenalectomized rats in this laboratory during the past 5 years it has been found that about 80 per cent of suprarenalectomized rats survive longer than 1 month. The mor-

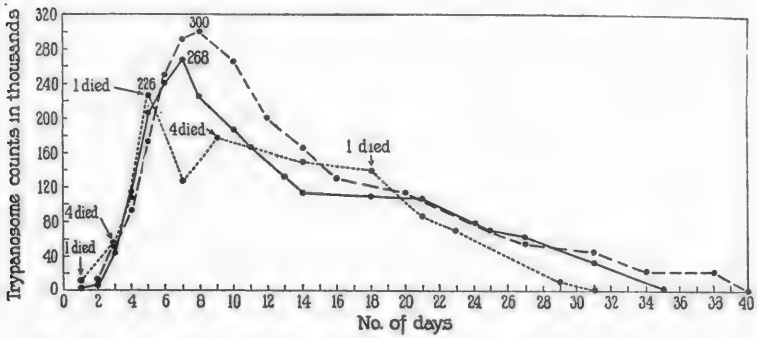

FIG. 1. The effect of bilateral suprarenalectomy, unilateral nephrectomy and traumatization of the suprarenal area on the course of *Trypanosoma lewisi* infection in albino rats.

Each point on the curve of the infection of the suprarenalectomized rats indicates the average count of the surviving animals on that day.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal———

Bilateral suprarenalectomy .....

Unilateral nephrectomy - - - - - - -

tality of 70 per cent to *T. lewisi* infection in the suprarenalectomized rats, therefore, indicates a severe drop in resistance.

The curve in Fig. 1 indicates the average daily trypanosome counts. Each point on the chart represents the average count of all the rats living on that day. The curve is practically the same as the curve of the composite average counts of the normal infected group.

An effort was made to reinfect the surviving rats but without success.

Pathology of the T. lewisi Infection in Suprarenalectomized Rats

A comparative study of the pathological changes in the suprarenalectomized group dying of *T. lewisi* infection and the group that had been normal prior to infection did not reveal any striking differences in cellular reaction.

TABLE II

Effect of Trypanosoma lewisi on the Weight of the Spleen in Normal and Suprarenalectomized Rats

Normal adult rats		Adult rats infected with *Trypanosoma lewisi*			Suprarenalectomized adult rats infected with *Trypanosoma lewisi*				Suprarenalectomized uninfected adult rats		
No. of rats	13	No.	Days after infection	Per cent weight of spleen	No.	Days after operation	Days after infection	Per cent weight of spleen	No.	Days after operation	Per cent weight of spleen
Per cent weight of spleen to body weight		1001	1	.170	1454	10	4	.687	1458	10	.265
		1002	3	.351	1462*	11	5	.728	1466	11	.288
Aver. wt.	.269%	1003	5	.852	1455	12	6	.821	1459	12	.245
Max. wt.	.448%	1004	7	1.195	1460	14	8	.645	1461	14	.251
Min. wt.	.104%	1005	9	1.316	1463	16	10	.832	1470	19	.224
Mean wt.	.268%	1006	11	1.554	1464*	17	11	.707	1471	20	.224
		1007	13	1.248							
		1008	15	.871							
		1009	18	.945							
		1010	20	.900							
		1011	22	.848							
		1012	25	.723							
		1013	28	.900							
		1014	32	1.000							
		1038	36	.670							
		1039	38	.560							

* Died.

Twelve rats were suprarenalectomized and six of these were infected with *T. lewisi* after 6 days. The other six were kept as suprarenalectomized controls. One rat in each group was killed every other day and the pathological changes were noted and compared with those in normal rats infected with *T. lewisi*. The ratio of the spleen weight to the body weight was noted in both groups and compared with the normal. Suprarenalectomy without infection does not cause an increase in the weight of the spleen (Table II), but histological studies made at frequent

intervals reveal a progressive hyperplasia of the follicles with no increase in the total volume. The hyperplasia of the follicles is part of a generalized lymph tissue and thymus hyperplasia evident throughout the body after suprarenalcetomy, as pointed out by Marine (21) and Jaffe (20).

The degree of splenomegaly in the suprarenalectomized and infected group was definitely less than in the unoperated infected group. On the tenth day after infection the spleen weighed 0.832 per cent of the body weight as compared with 1.554 per cent in the unoperated infected group (Table II). Though the degree of hypertrophy of the spleen is less, the character of the cellular response is the same in both groups. The reticular and endothelial elements are markedly distended with engulfed red blood cells and hemosiderin pigment. The pulp is congested and the follicles prominent.

In the second group of experiments 15 normal adult rats were infected with *T. lewisi* as described above and 1 month after all the trypanosomes had disappeared from the peripheral blood stream, the suprarenal glands were removed. After 5 days all were reinjected with *T. lewisi*. Reinfection did not occur in a single instance. It was not possible to overcome the acquired resistance by suprarenalectomy, regardless of the quantity of material used for reinjection.

Effect of Unilateral Nephrectomy and Traumatization of the Suprarenal Area on the Course of a Subsequent T. lewisi Infection

In an effort to control the factor of the operative procedure the left kidney was removed from eight adult rats by the posterior route and in five others the tissue about the suprarenal areas was injured. Six days after operation the rats were injected intraperitoneally with *T. lewisi* as described above. From Table I and Fig. 1 it is seen that the infection in these animals did not differ from that of normal rats. The average duration from the onset to the height of infection in the nephrectomized group is 8 days as compared with 7.5 of the normal group. The average number of trypanosomes at the height of infection was 330,000 per cubic millimeter, the range 100,000 to 890,000. All the rats survived the infection. The average duration of infection in the nephrectomized group was 26.7 as compared to 27.4 in the normal. The longest infection lasted 32 days.

One month after recovery all the rats were reinjected with *T. lewisi* but no reinfection was observed.

DISCUSSION

Bilateral suprarenalectomy lowers the resistance of adult albino rats to *T. lewisi* infection. In these rats the disease is fatal in almost 70 per cent of instances whereas of animals previously normal none died. Despite this high mortality the infection as characterized by the rate of growth of the trypanosomes does not differ essentially from the infection in normal rats. Neither the reproduction-inhibiting immune factor (Taliaferro) nor the trypanolytic immune factor is diminished by suprarenalectomy. Apparently the toxic effect of the infection is lethal in these animals. Suprarenalectomy further diminishes the degree of splenic response as estimated by the size of the spleen, but does not alter the reaction of the reticular and endothelial elements of the spleen or of the lymphoid tissue to the *T. lewisi* infection.

One infection with *T. lewisi* confers a permanent immunity in normal rats. It is of significance that suprarenalectomy does not break down the permanent immunity to *T. lewisi* infection acquired as a result of a first infection. This fact emphasizes an essential difference in the mechanisms of natural resistance and acquired resistance. The natural susceptibility of the rat to various toxins and poisons and infections is markedly increased by suprarenalectomy. But the acquired immunity established as a result of a first infection is uninfluenced by suprarenalectomy.

We have found in other studies that the *Bartonella muris* anemia of splenectomized rats cannot be transmitted to suprarenalectomized rats. It has been demonstrated in a previous communication that *Bartonella muris* anemia in the adult splenectomized rat represents a flaring up of a latent infection with the *Bartonella muris* virus and the development of the anemia is indicative of a depression in the acquired immunity of the rat to the virus (15). The failure of the suprarenalectomized rat to develop the anemia either spontaneously or after injection of anemic blood of splenectomized rats indicates that the suprarenal gland does not influence the mechanism of acquired immunity to this infection. This observation with the *Bartonella muris* infection and the fact that the permanent immunity to *T. lewisi*, acquired as a result of a previous infection, is uninfluenced by suprarenalec-

tomy are of significance. Once a humoral or cellular immunity is established to an infection this acquired resistance cannot be sufficiently depressed to permit of a second infection. Acquired resistance and natural resistance are dependent on different physiological processes in the organism and are not merely quantitative variations of the same process as is generally supposed.

SUMMARY AND CONCLUSIONS

Bilateral suprarenalectomy in rats lowers the resistance to a subsequent infection with *T. lewisi*. Almost 70 per cent of these rats die within an average period of 5.8 days after infection. The multiplication of the parasites in the circulating stream is not more considerable than in rats previously normal nor is the duration of the disease in the surviving rats any longer than in the normal group. Bilateral suprarenalectomy does not prevent the formation of immune substances to the parasites but appears to lower the natural resistance of the rat to the toxic effects of the protozoan infection. The acquired immunity to *T. lewisi* of normal rats as a result of infection is not broken down by subsequent suprarenalectomy. Unilateral nephrectomy does not affect the course of a subsequent infection with *T. lewisi*.

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FURTHER STUDIES ON T. LEWISI INFECTION IN ALBINO RATS*

I. The Effect of Splenectomy on T. lewisi Infection in Albino Rats and the Protective Action of Splenic Autotransplants

II. The Effect of Thymectomy and Bilateral Gonadectomy on T. lewisi Infection in Albino Rats

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(Received for publication, June 25, 1930)

The Effect of Splenectomy on the Resistance of Albino Rats to T. lewisi, and the Protective Action of Splenic Autotransplants

In studying the pathology of *T. lewisi* infection in normal rats it was noted that the spleen undergoes marked hyperplasia within a few days after injection of the organism (1). An effort was made to determine the rôle of the spleen in this infection by splenectomy at various intervals prior to infection with *T. lewisi* and to determine the effect of autoplastic splenic transplants on the infection in splenectomized rats.

The marked involvement of the reticular and endothelial cells of the spleen in the pathogenic trypanosome infections has lead to numerous investigations on the effect of splenectomy on the course of these infections. A review of the literature is given by Taliaferro (2).

Bradford and Plimmer (3) found that rats, dogs, cats and rabbits splenectomized before injection with *T. brucei* died earlier than the controls. Laveran and Mesnil (4) observed that splenectomy did not influence the course of *T. nagana* infection in the rat. Rodet and Vallet (5), as well as Sauerbeck (6), believe the spleen possesses striking trypanolytic power. Roux and Lacomme (7) treated three dogs infected with *T. nagana* with splenic extract of normal cattle and found

* Read before the International Congress of Microbiology, Paris, July 23, 1930.

that the parasites decreased in number but this may have been due to trypanolytic crises (Rodet and Vallet (5)). In a study on 2 splenectomized rats and 5 guinea pigs, Laveran and Thiroux (8) could find no effect of splenectomy on the course of infection with *T. brucei* and found no trypanolytic action of splenic extracts. They believed the spleen merely removed the débris after trypanolytic crises. Mutermilch (9) found that extracts of spleen produced lysis of *T. brucei in vitro*. Bone marrow and liver extracts were also effective but to a less degree, and extracts of other organs had no effect. From this he concluded that the hematopoietic organs are particularly trypanolytic. For a time it was thought that the spleen acted as a refuge for trypanosomes during the crises, and resistant forms were said to exist. But as Laveran and Mesnil (10) showed, such a theory was unwarranted as isolated trypanosomes could be demonstrated in the blood during the crisis by subinoculation methods. Taliaferro, Johnson and Cannon (2) found that splenectomy had no effect on the course of *T. equinum* infection in mice.

Taliaferro describes the formation of a humoral antibody in *T. lewisi* infections in the rat that inhibits the cell division of the parasite (11) but which does not kill them. Regendanz and Kikuth (12) confirmed these observations. They have further shown that in splenectomized rats division of the trypanosomes continued several days longer than in non-splenectomized controls and in some animals the infection ended fatally. They injected these rats with salvarsan prior to splenectomy to sterilize them of the *Bartonella* virus. Taliaferro, Cannon and Goodloe (13) found little effect if splenectomy was performed before injection of *T. lewisi* but if performed after injection the reproduction of the parasites again occurred, the adult forms beginning to divide. Splenectomy was more effective in the presence of *Bartonella* infection.

The rats of our stock are carriers of the *Bartonella* virus and splenectomy in these rats results in *Bartonella* anemia. We therefore had the opportunity of determining the influence of this infection on *Trypanosoma lewisi* infection.

The experiments were divided into three groups. In the first group 7 rats were infected 6 days after splenectomy at the height of the *Bartonella* anemia. A virulent infection resulted with rapid death of the rats within 3 to 7 days after infection. The average number of trypanosomes at the height of the infection reached 1,200,000 per cubic millimeter, about 4 times greater than the number of trypanosomes in the normal controls. The average duration of life was 11.6 days after injection of the trypanosomes. The height of the infection as estimated by the number of trypanosomes was reached at the time of death (see Table I and Fig. 1). We have found (14) that 100 per cent of our stock develop the *Bartonella*

anemia following splenectomy in from 4 to 10 days. The mortality from the *Bartonella* anemia in 50 splenectomized rats was 20 to 30 per cent. The anemia persists 2 to 3 weeks. The presence of the *Bartonella* infection markedly lowers the resistance of the animal to the *T. lewisi*. The *Bartonella* anemia probably destroys or injures the defensive mechanism (reticulo-endothelial elements?) and permits a more rapid reproduction of the organisms. In a previous communication (14) we have shown that an infection with *T. lewisi* in a rat with intact spleen permits the occurrence of the *Bartonella* anemia,

TABLE I

The Effect of Splenectomy on Trypanosoma lewisi Infection and the Protective Action of Autoplastic Splenic Transplants

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Splenectomy**.....	7	6	11.6	3	18	13	11.6	3	18	13	1200	349	2695	1000	0	100
Splenectomy.....	6	48	60	50	68	61	12.6	4	21	12	705	411	1125	600	90	10
Spl. trans.* and splen..	6	28	34.7	28	41	35	9	7	15	7	530	224	787	589	100	0
Unoperated adult normals.....	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0

** These rats died at height of infection.

* Transplants performed 4 weeks prior to splenectomy.

in mild form, as a result of injury to the reticular and endothelial elements of the spleen by this infection.

In the second group of experiments, 6 splenectomized rats were infected with *T. lewisi* 48 days after splenectomy and well after recovery from the *Bartonella* anemia. Of these, one died on the tenth day from the *T. lewisi* infection without a severe anemia. In the other 5 rats, the *T. lewisi* infection was very severe. The average number of the trypanosomes (see Table I) at the height of the infection was 705,000 per cubic millimeter or about twice that of the normal controls. The interval in days from the onset to the height of

the infection which represents the interval during which reproductive forms are present, averaged 12.6 days. Reproductive forms were present several days longer than in the normal rats. The duration of the infection in the rats was twice as long as the normal group, averaging 60 days.

This experiment indicates that the spleen plays an important rôle in the formation of the reproduction inhibiting factor and also the lytic factor in the infection; 48 days after splenectomy the infection with *T. lewisi* is less severe than in the early period. Apparently a compensatory mechanism has been established but this is less effective than is the normal splenic tissue in combating the *T. lewisi* infection.

In the third group of experiments, six rats in which splenic autotransplants had been introduced 4 weeks prior to splenectomy were infected with *T. lewisi* 28 days after splenectomy and 8 weeks after transplants had been made. The method of transplantation has been described (15). The transplants at the time the spleen was removed were insufficiently regenerated to prevent the occurrence of *Bartonella* anemia. The results of this experiment are indicated in Table I. The average number of trypanosomes at the height of the infection was 530,000 per cubic millimeter or midway between the splenectomized group infected 48 days after splenectomy and the normal group. The interval from the onset to the height of the infection was 9 days in this group as compared to 7.4 days of the normal rats and 12.6 days of the splenectomized rats without transplants. The duration of the infection in these rats was practically the same as in the normal group. The transplants reduced the severity of the infection of splenectomized rats to *T. lewisi*. Though they did not protect the rat against *Bartonella* anemia 4 weeks after they were implanted, the transplants at the end of 8 weeks, or 4 weeks after splenectomy, had regenerated sufficiently to raise the resistance of the animal to *T. lewisi*. Histological examination of these transplants (15) revealed complete regeneration of all elements of the spleen. They appeared as small spleens in the abdominal wall.

Reinfection in Splenectomized Rats.—An attempt was made to determine the rôle of the spleen in the acquired immunity of the rat to reinfection with *T. lewisi*. Ten normal rats were infected with *T. lewisi*. One month after recovery the spleens were removed from these rats.

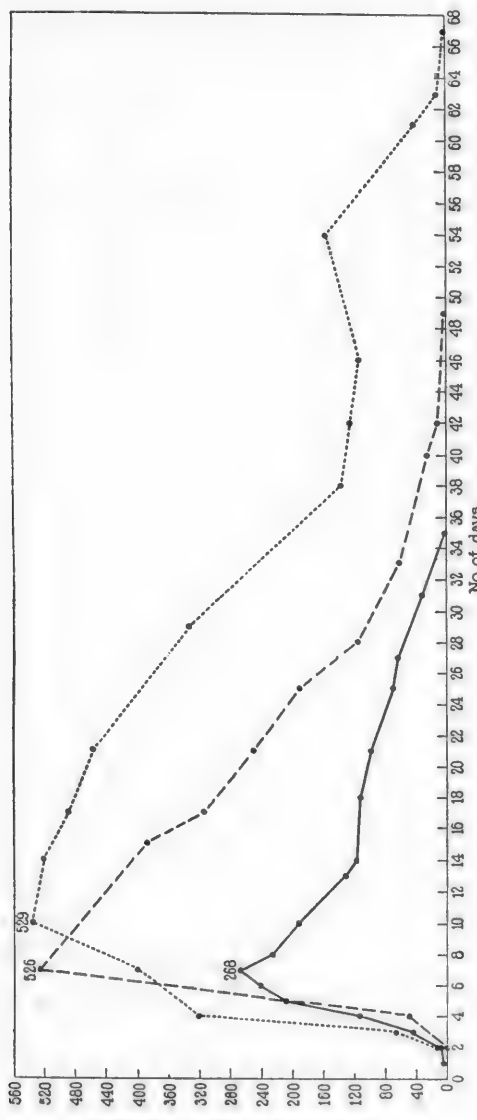

FIG. 1. The effect of splenectomy, and of splenectomy in rats with autotransplants of splenic tissue, on the course of *T. lewisi* infection in adult albino rats.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal———

Splenectomized rats infected 48 days after operation . . . . .

Splenectomized rats with splenic autotransplants performed 4 weeks prior to splenectomy and infected 24 days after splenectomy— — — —

Six days later all were reinjected with *T. lewisi*. In only one instance did they persist in the blood for a period of 48 hours. No developmental forms appeared. Repeated injections of *T. lewisi* were made, but no reinfection occurred.

DISCUSSION

Our observations are consistent with the findings of Regendanz and Kikuth (12), who studied the effect of splenectomy on the course of *Trypanosoma lewisi* infection in rats. Prior to splenectomy, their rats were injected with salvarsan to free them from the *Bartonella* virus. They found that the infection in the splenectomized rats was more severe than in the normal rats. They did not, however, make quantitative estimates of the course of the infection. The experiments reported in our communication indicate that the spleen plays a very important rôle in the defense mechanism to *T. lewisi*. The capacity of the rat to produce both the immune substance which inhibits the reproduction of the trypanosomes (reproduction-inhibiting immune factor) and the trypanolytic immune substance are markedly reduced by the removal of the spleen, even though the rats are not infected with *T. lewisi* until 7 weeks after the operation. This supports the contention of Taliaferro (2) that the reticulo-endothelial elements of the spleen are probably the main source of these immune substances.

The fact, however, that removal of the spleen does not influence the acquired immunity to *T. lewisi* following a first infection suggests that the immune defense mechanism may be a humoral one. In *Bartonella* infection on the other hand immunity may be a cellular one, dependent on the reticular and the endothelial elements of the spleen. In this disease an acquired immunity is broken down by splenectomy, but not by other procedures such as thymectomy, suprarenalectomy or gonadectomy. In trypanosomiasis the acquired immunity is a humoral one and hence is difficult to destroy. Complement fixing antibodies are present in the blood of rats for months after recovery from *T. lewisi* but no protective immune substances have thus far been demonstrated in the blood of rats either during the infection with *Bartonella* anemia or following it (Ford).

The conflicting results of investigations on the effect of splenectomy

on the pathogenic trypanosome infections is due in part to the difference in the species of experimental animal used. The spleen of the rat or mouse is considerably larger in proportion to the body weight than that of the rabbit or guinea pig. Taliaferro (2) suggests that the quantity of the reticular and endothelial cells in the spleen of the rabbit and guinea pig represents only a small part of the reticulo-endothelial tissue of the body. He believes that splenectomy in these animals therefore has little effect on the course of the trypanosome infections. This assumption however is based on insufficient evidence. It is possible that the relative quantity of hemolymph tissue in these different animals may bear some relation to the severity of the effects of splenectomy. Though it is still a debated question the true hemolymph nodes can probably be considered as accessory splenic tissue. The quantity of this tissue in the rat is extremely small. Macmillan (16) was able to find only 4 hemolymph nodes in the rat, 2 just above the kidney near the midline and 2 at the apex of the lungs in the posterior mediastinum. The rabbit and guinea pig, the sheep and the goat have relatively much more hemolymph tissue. The domestic pig on the other hand is said to have no true hemolymph nodes as determined by lymphatic injection, though many "red" nodes (Meyer (17)). Warthin (18) has demonstrated in the sheep and the goat a marked hypertrophy of the hemolymph nodes within the first few weeks following splenectomy, and considers these nodes as accessory erythropoietic tissue. It is possible that the hemolymph nodes in splenectomized animals assume a specific protective action which is normally a function of splenic tissue.

The Effect of Thymectomy in Young and Adult Rats on T. lewisi Infection

The effect of thymectomy on the course of *T. lewisi* infection in young and adult rats was studied. The rats were divided into two groups. In the first group of experiments 20 6-week old albino rats were used. Ten of these were thymectomized and 10 were kept as normal controls. In the second group, 10 adult, 3-month old rats were thymectomized. One week after operation, the rats of both groups were infected with *T. lewisi*.

Method.—Ether anaesthesia was used. The skin over the sternum was incised vertically and retracted. A purse string suture was put through the pectoral muscles, laterally and superiorly through the loose areolar tissue of the neck and through the submaxillary gland on one side. A similar purse string suture was put through the skin. The sternum was cut with small dull pointed scissors in the midline from above downward for a distance of 1.5 cm., care being taken to cut exactly in the midline to avoid the great vessels. The thymus was exposed, rapidly removed with fine mouse tooth forceps from below upward, and the purse string sutures were rapidly tied. If the chest is allowed to remain open longer than a few seconds the animal may succumb. If the rat survives the operation, it makes a rapid recovery.

The results of this experiment are tabulated in Table II and in the curves of the average daily counts of the trypanosomes in the circulating blood (Fig. 2). The course of the infection in 6-week old rats was slightly different from that of normal rats. The average number of trypanosomes at the height of infection was 316,000 per cubic millimeter as compared with 337,000 in the adult normal rats (see Paper VI, same issue of the journal (1)). The height of the infection was reached, however, somewhat earlier. In the adult normals the interval between the onset and the height of infection is 7.5 days, whereas in the younger group the interval between the onset and the height of infection is 6.4 days. The duration of the infection in the young rats is somewhat less than in the adult rats—22 days average duration in the young as compared to 27.4 days in the adult group. Thymectomy in the young rat has a decidedly beneficial effect on the course of the infection. The average number of trypanosomes at the height of infection is 176,500 per cubic millimeter and the interval between onset and the height of infection is 5 days. In the curve of the daily average counts the height of infection is reached on the 4th day and the average count on this day is 137,000. In the curve of the daily counts of the control young rats, the height of infection is on the 7th day and the average count on this day is double that of the thymectomy group, 273,000. The duration of the infection of the thymectomized rats is decidedly shorter than in the normal young controls. The average duration in the young controls is 22 days, and in the thymectomized young rats, 16.6 days.

The course and duration of the *T. lewisi* infection in adult thymectomized rats was likewise somewhat shorter than in adult normal rats.

The average number of trypanosomes at the height of infection was 241,000 and the interval between the onset and the height was 6.6 days. In the controls the average number of trypanosomes at the height of the infection was 337,000 and the interval from the onset to the height of infection was 7.5 days. The duration of the infection in the thymectomized adult rats is 19.1 days as compared with an average of 27.4 days in the control group.

Thymectomy has a favorable effect on the course of a subsequent infection with the *Trypanosoma lewisi* infection in both young and adult rats, but especially in young rats. The duration of the infection is decidedly less and the intensity of the infection is diminished. Developmental forms are observed during a shorter period and abortive infections are more frequently observed. The removal of the thymus, particularly in the young, has a stimulating effect on the formation of immune substances that inhibit the reproduction of the trypanosomes and have a stimulating effect on the formation of trypanolytic substances of the serum.

Effect of Unilateral and Bilateral Gonadectomy on T. lewisi Infection

Unilateral gonadectomy was performed on six adult albino rats and bilateral gonadectomy on six rats. One week after operation the rats were infected with *T. lewisi.*

Method.—Under ether, a median incision was made in the abdominal wall. The testes were gently drawn into the abdominal cavity by gentle traction on the spermatic cord. The pedicle was ligated and the testes were removed. Care was taken to avoid unnecessary manipulation of the spermatic cords. The abdominal wound was sutured. The rats recovered rapidly from the operation.

The results of this experiment are given in Table II and in Fig. 2. Unilateral gonadectomy in the adult rat has no effect on the trypanosome infection. The average number of trypanosomes at the height of infection is 276,000, reached in an average interval of 6.6 days as compared with the average number of trypanosomes at the height of the infection 337,000 in an average interval of 7.4 days. In the curve of the daily average counts the height is reached on the 7th day with 236,000 per cubic millimeter as compared with the normal 268,000 on the 7th day. Unilateral gonadectomy does not influence the course of a subsequent *Trypanosoma lewisi* infection.

TABLE II

Effect of Thymectomy and Gonadectomy on Trypanosoma lewisi Infection

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Thymect. in adults	10	6	19.1	7	27	22	6.6	4	8	8	241	60	570	196	100	0
6 week thymect.	10	—	16.5	6	24	20	5	4	6	5	176	60	315	160	100	0
Unilat. gonadect.	6	6	28	17	32	27.5	6.6	5	9	6	276	140	428	290	100	0
Bilat. gonadect.	6	6	26.5	25	31	25.5	7.2	7	9	7	889	550	1186	930	100	0
Unoperated adult normals	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0
Unoperated 6 week normals	10	—	22	17	27	22	6.4	4	8	7	316	60	570	225	100	0

TABLE III

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Bilat.*** supraren.	12	6	5.8	2	19	5	4.2	2	8	4	220	10	640	212	—	—
Bilat. supraren.	6	6	25	12	31	26.5	8.6	5	11	9	338	160	640	305	33	67
Splenectomy**	7	6	11.6	3	18	13	11.6	3	18	13	1200	349	2695	1000	0	100
Splenectomy	6	48	60	50	68	61	12.6	4	21	12	705	411	1125	600	90	10
Spl. trans.* and splen.	6	28	34.7	28	41	35	9	7	15	7	530	224	787	589	100	0
Unil. nephrect.	8	6	26.7	6	45	32	8	6	10	8	330	100	890	290	100	0
Thymect. in adults	10	6	19.1	7	27	22	6.6	4	8	8	241	60	570	196	100	0
6 week thymect.	10	6	16.5	6	24	20	5	4	6	5	176	60	315	160	100	0
Unil. gonadect.	6	6	28	17	32	27.5	6.6	5	9	6	276	140	428	290	100	0
Bilat. gonadect.	6	6	26.5	25	31	25.5	7.2	7	9	7	889	550	1186	930	100	0
Trauma to supr. area.	5	6	24	16	28	25	6.4	5	8	6	380	166	700	300	100	0
Unoperated adult normals	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0
Unoperated 6 week normals	10	—	22	17	27	22	6.4	4	8	7	316	60	570	225	100	0

*** Died during infection.

** Died at height of infection.

* Transplants performed 4 weeks prior to splenectomy.

Bilateral gonadectomy decidedly depresses the resistance to *T. lewisi*. Though the average duration of the infection is essentially

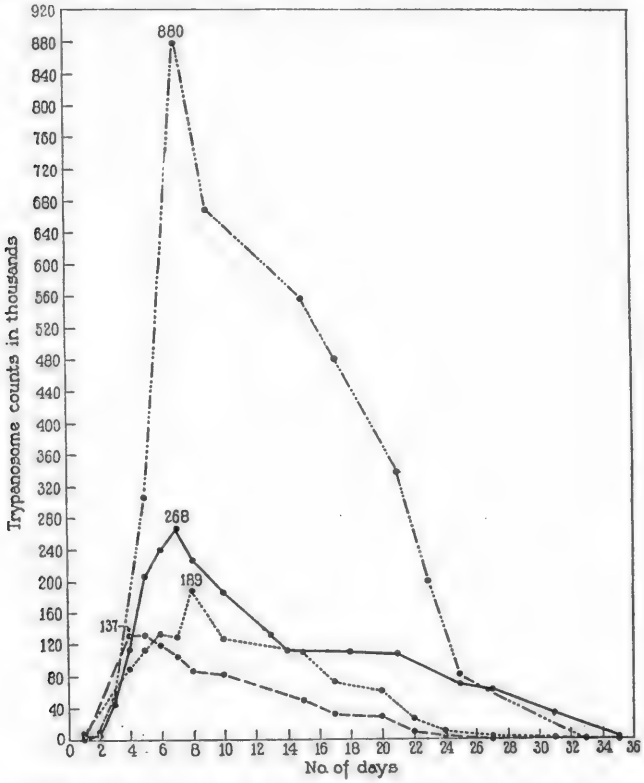

FIG. 2. The effect of bilateral gonadectomy and of thymectomy in young and adult albino rats on the course of *T. lewisi* infection.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal ————

Thymectomy in adult rat . . . . .

Thymectomy in young rats — — — —

Bilateral gonadectomy . .—. .—. .

the same as in the normal group, 26.5 days, the severity of the infection is markedly increased. The average number of trypanosomes

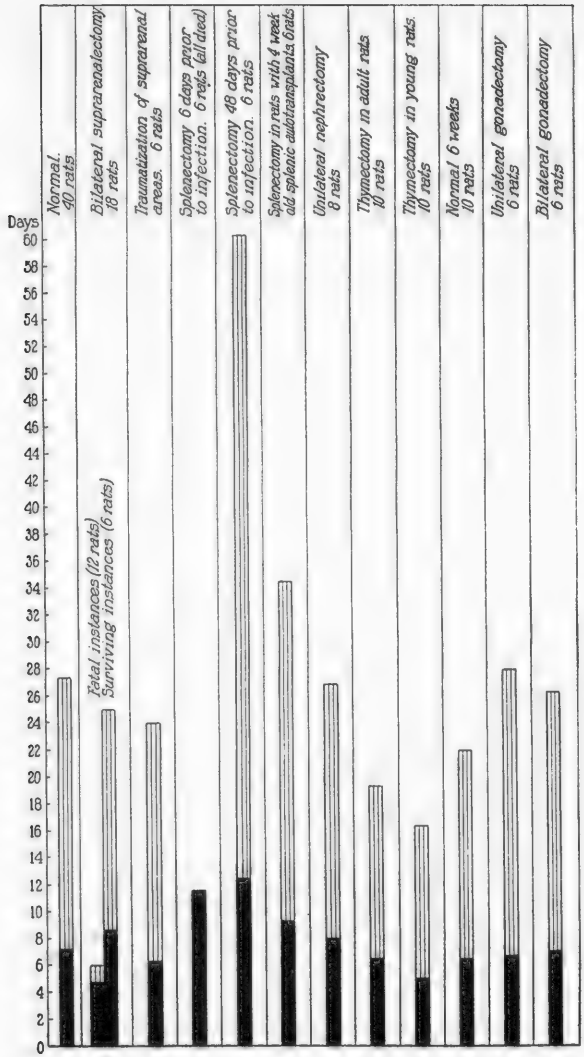

FIG. 3. The duration of infection with *T. lewisi* in normal rats, rats with bilateral suprarenalectomy, traumatization of the suprarenal area, unilateral nephrectomy, unilateral gonadectomy, bilateral gonadectomy, young and adult rats with thymectomy, rats in which splenectomy was performed 6 days prior to infection, rats in which splenectomy was performed 48 days prior to infection, rats with splenectomy n which splenic autotransplants were performed 4 weeks prior to splenectomy.

The solid portion of the bars represents the interval in days from the onset of the infection to the height of the infection.

The entire column represents the average duration in days of the infection.

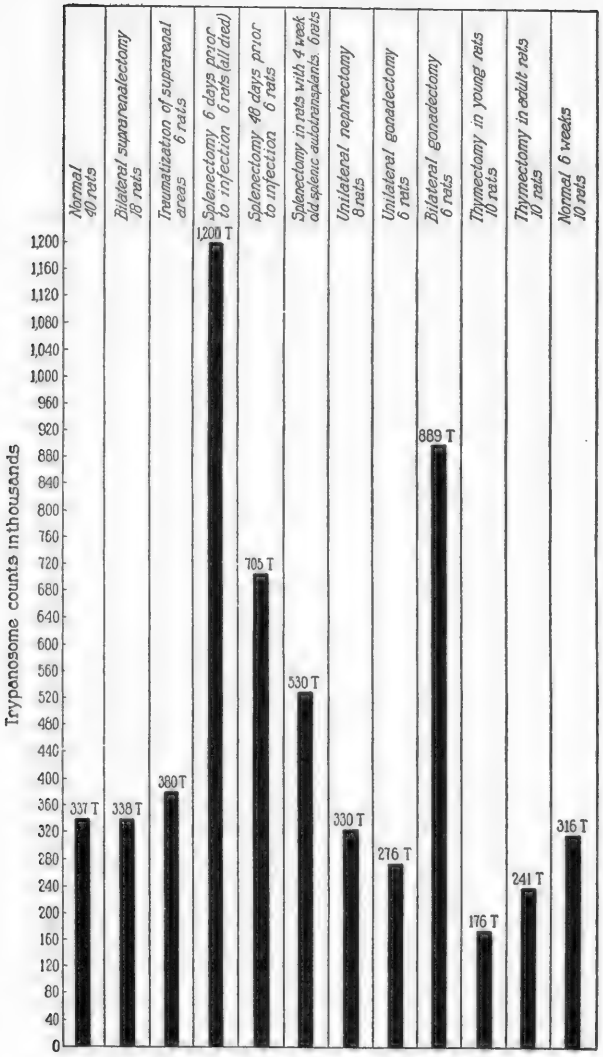

FIG. 4. The number of trypanosomes at the height of infection in rats with bilateral suprarenalectomy, traumatization of suprarenal area, unilateral gonadectomy and bilateral gonadectomy, thymectomy in young rats, thymectomy in adult rats, splenectomy in rats infected 6 days after operation, splenectomy in rats infected 48 days after operation, splenectomy in rats in which splenic autotransplants had been performed 4 weeks prior to splenectomy.

The bars represent the average number of trypanosomes at the height of infection with *T. lewisi*.

at the height of infection is 889,000 or almost three times as many as the normal group. The curve of the daily average counts reached a peak of 880,000 on the 7th day as compared with 268,000 on the 7th day in the normals. In 3 of the rats with bilateral gonadectomy the count exceeded 1,000,000 per cubic millimeter. Splenectomy resulted in similarly high counts. However, the disease in the bilaterally gonadectomized rats did not last longer than in the normals nor were there any fatalities. We must conclude that removal of the gonads interferes with the formation of the immune substance which inhibits reproduction of the trypanosomes, but does not interfere with the formation of the trypanolytic antibodies. In 4 instances in this group the disease terminated sharply by crisis, which is less frequently the case in normal rats.

Thymectomy in young rats diminishes the severity of the infection and shortens its course. Bilateral gonadectomy in the adult increases the severity of the infection but does not influence the duration of the infection.

SUMMARY

T. lewisi infection in normal adult 3 month old albino rats raised from a single stock and maintained under identical conditions was studied. Daily quantitative estimates of the trypanosomes in the circulating blood were made and the course of the infection was studied. Bilateral suprarenalectomy in rats lowers the resistance to a subsequent infection with *T. lewisi*. About 70 per cent of these rats die in an average period of 5.8 days after injection. The multiplication of the parasites, in the circulating stream, however, is not more considerable in the suprarenalectomized than in the previously normal rats, nor is the duration of the disease in the surviving rats any longer than in the normal group. The removal of the suprarenal glands does not alter the immune reaction to the parasite, but lowers the natural resistance of the animal to the toxic effects of the protozoan infection. Bilateral suprarenalectomy does not lessen the immunity of rats recovered from *T. lewisi* infection to subsequent infection. Unilateral nephrectomy does not influence the course of a subsequent infection with *T. lewisi* infection. The mortality of splenectomized rats from *Bartonella muris* anemia increases from 30 to 100 per cent following

the injection of *T. lewisi* at the height of the anemia 7 days after splenectomy. *T. lewisi* infection 48 days after splenectomy that is to say at a time when the *Bartonella* anemia is no longer present produces a more severe infection than in normal rats. The number of trypanosomes at the height of infection averages 3 times the ordinary and the infection endures twice as long. Both the immune substance that inhibits the reproduction of the parasite and the lytic factor are markedly depressed. Splenic autotransplantation performed 4 weeks prior to splenectomy raises the resistance of rats to a subsequent *T. lewisi* infection. Thymectomy in 6 week old rats diminishes the severity of a subsequent trypanosome infection and shortens its course. Both the formation of the immune substance which inhibits reproduction of the trypanosomes and formation of trypanolytic antibodies are stimulated by this procedure. In the adult rat thymectomy shortens the course of the infection but the severity is only slightly diminished. Bilateral gonadectomy in the adult increases the severity of the infection. The number of trypanosomes at the height of the infection is almost three times the normal. However, the duration of the infection is the same as in the normal rats. The reproduction-inhibiting factor is depressed by bilateral gonadectomy but not the trypanocidal factor. Unilateral gonadectomy does not influence the infection.

We wish to thank Dr. David Marine for his helpful criticism throughout the course of this work.

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BACTERIUM GRANULOSIS IN RELATION TO TRACHOMA: ITS RECOVERY FROM EXPERIMENTALLY INFECTED MONKEYS AND FROM HUMAN TRACHOMA

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PLATE 26

(Received for publication, June 13, 1930)

Since Noguchi's report in 1928 (1), reports have come from laboratories in various parts of the world (Stepanowa and Azarowa in Kharkov (2), Finnoff and Thygeson in Denver (3), Addario in Palermo (4), Kendall in Chicago (5), Tilden and Tyler (6) and Olitsky and Tyler (7) in New York, and Lumbroso in Tunis (8)) of the isolation of *Bacterium granulosis* from trachoma and the reproduction in monkeys of granulomatous lesions of the conjunctiva such as were originally secured by Noguchi. It has now become desirable to assemble as many strains as possible of both European and American origin, in order that a comparative study can be carried out. Since the cultures survive for many weeks, even months, on the semisolid leptospira medium at ordinary temperatures, when sealed in ampoules, such a study is entirely feasible.

Certain animals described in Noguchi's monograph are still alive, $2\frac{1}{2}$ years after inoculation, and continue to show the granulomatous lesions. The lesions in the chimpanzee "Louisa," which reached their maximum development within a period of 8 months, have since remained stationary. Of the *Macacus rhesus*, No. 80 shows, at the end of $2\frac{1}{2}$ years, complete healing, with scar tissue, in the left eye (Fig. 2), while the right lid still carries a few follicles. Two *Macacus rhesus* (Nos. 5 and 6) inoculated from *Macacus* 73 in February, 1928, continue to have pronounced lesions. In June, 1929, No. 5 showed a spontaneous exacerbation, with marked redness and lacrimation, the cornea becoming diffusely cloudy (Fig. 3).

The present report deals with the following experiments: (1) the further transmission (a) by animal passage, and (b) by inoculation of cultures, of the Albuquerque Strain 1, isolated by Noguchi from Indian trachoma in 1926; (2) the recovery of the organism from monkey lesions, in their early stages, and also after they had persisted for a year or longer; (3) the isolation of new strains from cases of Indian trachoma, and (4) comparative cultural studies of the old and new strains and viability tests of the bacterium. The isolation of new strains was made possible through the cooperation of Dr. F. I. Proctor and the U. S. Indian Service. For clinical assistance we are indebted to Drs. Polk Richards and J. F. Lane, of the Indian Service.

1. Experiments with Albuquerque Strain 1 (Noguchi)

(*a*) *Transmission by Animal Passage.*—

The transfer of the infection from monkey to monkey was accomplished by means of tissue suspensions, the entire affected tarsal conjunctiva being removed, under ether anesthesia, ground in a mortar with a minimum quantity of isotonic sodium chloride solution, and injected subconjunctivally and also applied to the scarified conjunctiva, as described by Noguchi.

The first passage from two monkeys injected with cultures by Noguchi in June, 1927, (Nos. 72 and 73) gave positive results in most of the animals inoculated (Table 1). The only exception was an experiment (9) in which the suspension from the right conjunctiva of No. 72 was distributed among 15 animals. This diluted material failed in all but one instance to produce infection. With later passages from the same series the incubation period became longer and the positive results less frequent, and further transmissions were abandoned in favor of experiments with cultures.

(*b*) *Transmission by Means of Cultures.*—Although the original cultures had been carried on artificial medium for a period of $2\frac{1}{2}$ to 3 years, having been transplanted every 6 to 8 weeks, inoculation tests gave positive results (Fig. 4) in about one-third of the animals (Table 2), showing that *Bacterium granulosis* may retain its pathogenicity for long periods of artificial cultivation.

TABLE 1

Transmission of Albuquerque Strain 1 by Passage

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1927		*days*			
1	Dec. 9	Conj. susp. No. 72 N**	13	++++	++++	Lesions persisted 1½ years. Died
2	"	"	13	++++	+	Lesions persisted 1 year. Right eye negative until 11 months after inoculation
	1928					
3[1]	Feb. 10	Conj. susp. No. 73 N**		−	−	
4[1]	"	"		−	−	
5[1]	"	"	66	++++	++++	Lesions still present (2 years). Cultures recovered
6[1]	"	"	45	++	++	Receded in 1 month
7[2]	"	"		−	−	
8[2]	"	"	45	++++	++++	Lesions on right still present. Cultures recovered
9[2]	"	"	33	++++	++++	Lesions persisted 20 months. Cultures negative. Died
10[2]	"	"		−	−	
11	"	"	66	+++	++++	Lesions still present after 19 months. Cultures recovered. Discarded
12	"	"	33	++++	++++	Lesions persisted 16 months. Discarded
13	"	"	139	+++	+++	Lesions persisted 8 months. Discarded

* Inoculations are always made into the left conjunctiva under novocain anesthesia (2 per cent).

** Numbers followed by N refer to monkeys inoculated by Noguchi, and protocols will be found in his report.

[1] Received 5 subcutaneous injections of heat-killed cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

[2] Received 5 intravenous injections of cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

TABLE 1—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1928		*days*			
14	Feb. 10	Conj. susp. No. 73 N**	66	+++	+++	Lesions persisted 14 months. Discarded
15	"	"		−	−	
16	Mar. 9	Conj. susp. No. 72 N		−	−	Reinoculated May 16, 1928
17	"	"		−	−	Died of tuberculosis May 16, 1928
18	"	"	24	++	+	Lesions persisted 15 months, but did not progress further
19	Apr. 13	Conj. susp. No. 2		−	−	
20	"	"		−	−	
21	"	"		−	−	
22	"	"		−	−	
16	May 16	Conj. susp. Nos. 86 N, 79 N, and No. 1	83	−	++++	Died of tuberculosis in 3 months
23	"	"		−	−	
24	"	"		−	−	
25	"	"		−	−	
26	"	"		−	−	
"June" chimpanzee	June 20	Conj. susp. "Venus" and 81 N	66	+++	++++	Lesions persisted 1 year; disappearing
31	"	"	48	−	++	Died in 4 months
32	"	"	132	+++	−	Lesions persisted 13 months and remained unilateral. Discarded
33	"	"		−	−	
34	"	"		−	−	

TABLE 2

Inoculations with Routine Subcultures of Bacterium granulosis (Strain 1 Noguchi)

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1928					
27	June 12	Cultures on horse blood and horse serum agar plus carbohydrates and on semi-solid medium, 4 and 13 days old	16 days	++++	++++	Lesions persisted 6 months. Cultures negative at 176 days, transmission positive
28	"			−	−	Discarded after 140 days
29	"			−	−	"
30	"			−	−	"
"Paulina" chimpanzee	Aug. 8	Similar cultures, 4 days old	16 days	+	+	Few follicles at border of tarsus. Did not progress further
35	"	"		−	−	
36	"	"		−	−	
37	"	"		−	−	
38	"	"		−	−	
"Julia" chimpanzee	Nov. 21	Similar cultures, 4 and 15 days old	42 days	++	++	Few granules at border of tarsus, and on tarsal plate. Did not progress further
41	"	"		−	−	Discarded after 6 months
42	"	"		−	−	Discarded after 7 months
43	"	"	5 mos.	++	++	Lesions receded after 2 months
44	"	"		−	−	Discarded after 117 days
	1929					
61	Jan. 24	Similar cultures, 6 days old	11 days	++++	++++	Lesions progressed for 8 months. Cultures positive at 4 months. Few follicles remaining after 11 months. Discarded
62	"	"	11 days	++	++	Transient lesions, disappeared in 4 months
63	"	"	11 days	+++	+++	Died in 3 months

2. *Recovery of Bacterium granulosis (Albuquerque Strain 1) from the Monkey Lesions*

The material used for isolation experiments was similar to that employed for direct passage. The basic medium was 2 per cent nutrient agar, adjusted to pH

TABLE 3

Recovery of Bacterium granulosis from Monkey Lesions

Monkey No.	Inoculated		Incubation	Result		Date cultured	Result
	Date	Material		Left	Right		
	1927		*days*				
81 N	Oct. 13	Conj. susp. "Louisa"	89	++++	++++	Dec. 5, 1928 (390 days)	+
	1928						
5	Feb. 10	Conj. susp. 73 N	66	++++	++++	Dec. 5, 1928 (299 days)	+
27	June 12	Strain 1 cultures	16	++++	++	Dec. 5, 1928 (176 days)	−
8	Feb. 10	Conj. susp. 73 N	45	++++	++++	Jan. 31, 1929 (356 days)	+
12	"	"	33	+++	+++	Jan. 31, 1929 (356 days)	−
46	Dec. 5	Conj. susp. No. 5	47	+++	+++	Jan. 31, 1929 (57 days)	+
9	Feb. 10	Conj. susp. No. 73 N	33	++++	++++	Mar. 20, 1929 (405 days)	−
11	"	"	45	++++	+++	Mar. 20, 1929 (405 days)	+
18	Mar. 9	Conj. susp. No. 72 N	24	++	+	Mar. 20, 1929 (377 days)	−
	1929						
61	Jan. 24	Strain 1 cultures	11	++++	++++	Apr. 5, 1929 (71 days)	+
	1927						
Chimpanzee "Louisa"	May 20	Conj. susp. chimpanzee "Kitty" (3d passage)	28	++++	++++	May 8, 1930 (1083 days)	+

7.4, to each 100 cc. of which was added 12 cc. of defibrinated horse blood and 5 cc. of a Berkefeld V filtrate of a mixture of 10 per cent dextrose, 10 per cent saccharose, 2.5 per cent mannose, 2.5 per cent levulose, and 2.5 per cent inulin. The conjunctival suspensions were smeared over the freshly prepared plates, which were then strapped with adhesive tape and incubated at 28°C. for 3 to 6 days.

TABLE 4

Inoculation of Cultures Recovered from Monkeys

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1928					
55	Dec. 12	No. 81 N cultures, I gen.,		−	−	Discarded after 177 days
56	"	52 hrs. old, semisolid medium	147 days	++++	++++	Lesions persisted 9 months
57	"			−	−	
58	"			−	−	
51	"	No. 5 cultures, I gen. 52 hrs. old, semisolid medium	100 days	++++	++++	Marked congestion and secretion noted 65th day of disease (Fig. 5). Cultures (secretion) negative
52	"	"		−	−	
53	"	"		−	−	
54	"	"		−	−	
	1929					
60	Jan. 16	No. 5 cultures, 12 days old, blood agar and semisolid	142 days	++++	++++	Lesions persisted 4 months. Still marked when animal was discarded
59	"	"		−	−	
64	Jan. 24	No. 5 cultures, 6 days old, blood agar and semisolid	11 days	+++	+++	Lesions progressed for 6 months
65	"	"	11 days	+++	+++	"
66	"	"	11 days	+++	+++	"
67	"	No. 81 N cultures 6 days,	22 days	+++	+++	Lesions persisted 6 months
68	"	blood agar and semisolid	11 days	+	+	Died of pneumonia, 4 months
69	"	"	11 days	+++	+++	Receding after 7 months. Discarded
45	Apr. 3	No. 8 cultures I, II, III gen.		−	−	
47	"	"	35 days	+	±	Negative after 5 months
48	"	"		−	−	

TABLE 4—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929					
52	Apr. 3	No. 46 cultures I, II, III gen.	35 days	++++	++++	Lesions persisted 7 months
53	"	"		−	−	
54	"	"	65 days	++++	++++	Lesions persisted 7 months
70	"	No. 61 cultures III gen., blood agar and semi-solid	1–2 mos.	++	−	Transient lesions
71	"	"	21 days	++++	++++	Lesions persisted 6 months. Discarded
72	"	"		−	−	
73	"	"	21 days	++++	++++	Lesions persisting (10 months)
74	May 17	No. 11 cultures blood agar and semisolid IV gen.		−	−	Discarded after 4 months
75	"	"		−	−	"
76	"	"		−	−	"
77	"	"		−	−	"

Cultures were recovered from 6 of 10 *Macacus rhesus* cultured, and from the chimpanzee "Louisa." With one exception, these animals had all been infected by passage from other monkeys; one *rhesus* had received cultures. The periods elapsing between inoculation and culture varied from 57 days to nearly 3 years (1083 days), and the length of this period seems to have had no relation to the success of recovery. The recovery of the organism from the chimpanzee "Louisa" 3 years after inoculation shows that the organism may persist in the experimental infection, as in the human disease, for a long period of time.

The cultures recovered in some instances proved highly virulent (Fig. 5).

3. Recovery of Bacterium granulosis from Human Trachoma Lesions

(*a*) *Fort Defiance Cases.*—In May, 1929, an expedition was made to Fort Defiance, Arizona, where is situated one of the two special schools

which the United States Government maintains for the segregation of Indian children having trachoma. Five children who had been receiving daily treatment with copper sulfate or silver nitrate and two with advanced trachoma who had never received any treatment were selected for study.

Case 1. K., female, probably between 7 and 9 years old. Has been in the school 1 year; had advanced trachoma when she came. Scar tissue present. Treated with silver nitrate daily from Sept. 1928 to Jan. 1929, since then with copper sulfate (until May 16). Tissue taken May 23, 1929.

Case 2. A. L., female, about 7 years old. Has been in the school 2 years. Had advanced trachoma when she came. Scar tissue present. Treatment as in Case 1. Tissue taken May 23, 1929.

Case 3. N. T. Y., male, about 8 years old. Has not been in the school and has had no treatment. Mostly scar tissue; few follicles along edge of tarsus. Portion of tissue showing follicles taken.

Case 4. S., male, 7 years old. Conjunctivae covered with follicles, not much scar tissue. Has not been in the school and has had no treatment. Duration of disease unknown.

Case 5. A. L., female, 12 years old. From St. Michael's school. Disease probably of 3 to 4 years duration. Upper lids show mostly scar tissue, but lower lids show follicles; tissue taken from lower lids. Treatment irregular.

Case 6. K. A., female, 10 years old. Trachoma of 5 years duration. Mostly scar tissue; few follicles. Treatment as in Case 1.

Case 7. L. W., female, 9 years old. Mostly scar tissue, few small follicles. Duration of disease unknown. Treatment as in Case 1.

The eyes were washed with sterile saline, and the follicles were removed by Dr. Richards after novocain anesthesia (2 per cent). The medium used was the same as that employed for cultivation from the monkey conjunctiva, except that some plates were made with human blood. The plates, which had been brought from New York in a humidor and protected to some extent from drying by being strapped with adhesive tape, were again closed with adhesive after inoculation. They were transported to New York in the humidor and were 9 days old when examined.

Cultures of *Bacterium granulosis* were obtained from Cases 3 and 4, *i.e.*, from the two cases which had received no treatment. The human blood medium appeared to be as satisfactory as that made with horse blood. The cultures were typical of *Bacterium granulosis* morphologically and culturally and induced the characteristic conjunctival lesions in monkeys (Table 6). Subsequently their action on carbohydrates was determined and found to conform with that of the Albuquerque Strain 1 of Noguchi.

(*b*) *Santa Fé and Albuquerque Cases.*—A second expedition to the Indian Schools was made in September, 1929. We are indebted to the Indian Service for providing facilities for the preparation of culture medium at the U. S. Indian Hospital in Santa Fé. No advanced untreated cases of trachoma were available for cultural study either at Santa Fé or at Albuquerque, and experiments with treated cases

TABLE 5

Monkey No.	Date	Culture inoc.		Incubation period	Result		Remarks
		Age	Temp.		Left	Right	
	1929	*days*		*days*			
P-1	June 15	57	4°–6°C. 44 days	73	+++	+++	Lesions persisting (8 months)
P-2	"	"	"	"	+++	+++	Died after 144 days
P-3	"	"	"	"	+++	+++	Died after 127 days
P-4	Sept. 28	162	4°–6°C. 149 days		−	−	
P-5	"	"	"		−	−	
P-6	"	"	"		−	−	
	1930						
P-7	Feb. 10	297	4°–6°C. 284 days		−	−	
P-8	"	"	"	8	++	++++	
P-9	"	75	Room 69 days		−	−	
P-10	"	"	"	8	+	+	

proved negative. It is of interest in this connection that a recent report of the Indian Service (10) shows a marked decline in the incidence of trachoma among the New Mexico (Pueblo) Indians, owing apparently to the systematic treatment which is being carried out in the Pueblo villages.

(*c*) *Leupp Cases.*—Through the kindness of Doctors Proctor and Richards, in cooperation with Commissioner Rhoads, of the Indian

TABLE 6

Inoculation of Bacterium granulosis from Fort Defiance Cases

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
F. D. 1	June 10	Cultures I gen. 7 days semi-solid II gen. blood agar 4 days, Case 3	77	+++	++++	Lesions progressed for 3 months. Discarded 5 months
F. D. 2	"	"		−	−	Reinoculated with pooled strains Cases 3 and 4 after 5 months. ++ lesions in 35 days
F. D. 3	"	"	64	+++	++++	Began to recede after 2 months. Discarded 7 months after inoc.
F. D. 4	"	"		−	−	
Chimpanzee "May"	"	"		−	−	Reinoculated with pooled strains Cases 3 and 4 after 5 months. Negative
F. D. 5	"	Similar cultures. Case 4	132	+++	+++	Lesions progressed for 2 months. Discarded 1 month later
F. D. 6	"	"	64	+++	++++	Lesions progressed for 3 months
F. D. 7	"	"	117	+	+	Transient lesions
F. D. 8	"	"		−	−	
F. D. 9	July 27	Case 4 cultures III, IV, V gen.		−	−	Discarded after months
F. D. 10	"	7, 14, 19, 25		−	−	"
F. D. 11	"	days semisolid med. and blood agar		−	−	"
F. D. 12	Nov. 11	Pooled cultures. Cases 3 and 4 10 days semi-solid	35	++	+++	
F. D. 13	"	"	35	+	+	Died 78 days after inoc.

TABLE 6—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
Passage from F. D. 3						
F. D. 14	Aug. 16	Conj. susp. No. F. D. 3		—	—	
F. D. 15	"	"		—	—	
F. D. 16	"	"		—	—	
F. D. 17	"	"		—	—	

Service, a search for untreated cases of trachoma was made in the Navajo territory in Arizona, where systematic treatment has been less successfully applied because of the nomadic habits of the Navajos. It was found that at the Indian School at Leupp the treatment had not been carried out for at least 6 months, no physician having been on duty regularly. Thirteen cases were selected by Dr. Richards for study. Of these, 6 were cases of granular conjunctivitis of recent onset in which the diagnosis was not altogether certain, but in which there had been no treatment whatever, 5 were unquestionably trachoma, and 2 showed evidence of the beginning scar tissue formation, indicating that they were in all probability trachoma. The clinical data are presented in Table 7.

The follicular material used for inoculation of the culture media was removed by Dr. Richards, the eyes being first washed with sterile saline and the lids anesthetized with 0.5 per cent novocain. The usual medium was employed, *i.e.*, blood agar plates containing horse or human blood and a mixture of carbohydrates. The latter ingredient was the mixture originally used by Noguchi (1). It had been sterilized fractionally for 10 minutes on 3 successive days at 103°C. in a pressure cooker. There was some sedimentation on cooling, and only the clear supernatant fluid was added to the medium. The plates, which were prepared in Santa Fé the day previous to their use in Leupp, were strapped with adhesive tape after inoculation and brought back to New York in a humidor. They were not opened until arrival in New York, 9 days after inoculation. The weather conditions on the day of inoculation were unfortunate, in that high winds prevailed, and much sand and dust blew in through the windows and skylight of the operating room, where the cultures were made, hence the plates were in most cases nearly

overgrown with molds by the 9th day. A few, however, were less seriously contaminated, and colonies of *Bacterium granulosis* were found to be present. The follicle suspensions were brought back to New York and fresh plates inoculated.

TABLE 7

Case No.	Patient	Age	Diagnosis	Remarks	Culture
1	E. L.	7	Trachoma. Beginning scar tissue	Fairly recent case. Treated last year	−
2	M. N.	9	Doubtful (*i.e.*, no scar tissue)	May be beginning trachoma. Treated last year	−
3	P. T.	7	"	May be beginning trachoma. Treated last year	−
4	F. C.	14	Unquestionably trachoma. Scar tissue present	Old case. No treatment since last May	+
5	J. D.	10	"	Old case. Treated last year	+
6	H. W.	6	Recent case; some evidence of beginning scar tissue formation	Treated last year	+
7	M. C.	9	Recent case; some evidence of beginning scar tissue. Follicles in left eye only	Treated last year	−
8	B. H.	9	Unquestionably trachoma. Scar tissue present	No treatment since last May	−
9	H.	6?	Doubtful (*i.e.*, no scar tissue)	No treatment	+
10	I. M.	6?	"	" "	−
11	K. W.	7	"	" "	−
12	M. (Keems Canyon)	9	Unquestionably trachoma. Scar tissue present	" "	−
13	E. M.	10–11	Doubtful (*i.e.*, no scar tissue). Right eye only affected	" "	−

From the 4 cases which were regarded as unquestionably trachoma, *Bacterium granulosis* was obtained in 3. The organism was also obtained from Case 6, which showed evidence of early scar tissue forma-

tion, and from Case 9, which showed no scar tissue. The results of inoculation of the pooled cultures from Cases 5, 6, and 9 are recorded in Table 8. The lesions in Monkey L-6 are shown in Fig. 1.

4. Cultural and Biological Characteristics of the Various Strains

(*a*) *Viability.—Bacterium granulosis* remains viable for long periods on the semisolid ("leptospira") medium, and cultures 6 months old, kept at room temperature, with no other protection than the

TABLE 8

Inoculation of Bacterium granulosis from Leupp Cases

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
L-1	Nov. 22	Pooled cultures Cases 5, 6, and 9, I–II gen. semisolid med., blood agar plus carbohydrates, and hormone agar plus carbohydrates 48–72 hrs. old		−	−	
L-2	"	"	13	++	++++	++++ at 74 days
L-3	"	"	13	++	++	++ " " "
L-4	"	"		−	−	Died 13 days after inoc.
L-5	"	"	13	++++	++++	++++ at 74 days
L-6	"	"	13	++++	++++	++++ " " "

cotton plug, are readily transplantable. Even on a plain agar slant, on which the organism grows less readily, it has been found to remain transplantable for at least 49 days. It is probable that the bacterium lives as long as the medium contains a certain amount of moisture. Cultures kept in sealed ampoules for periods of 6 weeks to 10 months at 4° to 6°C., and for 69 days at room temperature, have proven viable and infective (Table 5).

(*b*) *Cultural Characteristics.*—The cultural characteristics of the strains of *Bacterium granulosis* thus far isolated have been practically

constant. In Tables 9 and 10 are shown the results of fermentation tests of the original strain and of the six strains isolated by us from Indian trachoma, as well as seven strains isolated by Finnoff and Thygeson (3) and one by Kendall (5), which these workers kindly sent us.

The tests recorded in Table 9 were made on Hiss serum water medium containing 1 per cent of the respective carbohydrates and litmus (about 0.01 per cent of a 25 per cent solution) as indicator, to which were added 10 per cent horse serum and 0.2 per cent horse hemoglobin; those shown in Table 10 were made on Dunham's peptone water containing 1 per cent carbohydrate and 1 per cent Andrade indicator (0.5 per cent acid fuchsin to which normal sodium hydroxide is added until the dye is decolorized). With the latter media a definite acid reaction is evident in 3 to 5 days, the lactose being the only one of the carbohydrates affected which shows no reaction until after this period. The use of Durham tubes shows that no gas is formed in any of the carbohydrate media.

Bacterium granulosis reduces nitrates to nitrites but forms no indole. It does not liquefy gelatin.

It has been found that after a few subcultures, all the strains of *Bacterium granulosis* grow well on nutrient agar (veal infusion agar containing 1 per cent of Witte's peptone and 0.5 per cent sodium chloride). The medium must be freshly prepared. Growth is more abundant if the agar is enriched by the addition of a carbohydrate mixture and horse blood or rabbit hemoglobin. A number of carbohydrates has been added individually to the horse blood agar medium in a concentration of 2 per cent; of these, media containing dextrose gave the best growth.

(*c*) *Reaction to Gram's Stain.*—Since *Bacterium granulosis* failed to retain an appreciable amount of the Gram stain (Sterling's gentian violet) when decolorized for 2 minutes and counterstained with dilute fuchsin, Noguchi regarded it as Gram-negative. It may be noted that Morax (11) regards it as Gram-positive, and since he apparently does not make use of a contrast stain in his technique, his point of view is comprehensible. However, after a contrast stain, even one as weak as Bismarck brown, no Gram stain can be detected in young, actively growing cultures. In old cultures, or cultures grown under unfavorable conditions (*e.g.*, in dextrose broth), numerous bizarre forms are seen which may retain the Gram stain. A form with an equatorial

swelling, suggesting a central spore, is not uncommon in such cultures and usually stains Gram-positive, especially in the central portion.

(*d*) *Effect of Heat.—Bacterium granulosis* is rather sensitive to heating, being killed by exposure for 10 minutes to a temperature of 57°C. The thermal death point was tested by submerging sealed ampoules

TABLE 9

Fermentation Tests. Hiss Serum Water Medium

	F. D. 3	F. D. 4	L. 5	L. 6	L. 9	Kendall
Dextrose	+	+	+	+	+	+
Levulose	+	+	+	+	+	+
Mannose	+	+	+	+	+	+
Saccharose	+	+	+	+	+	+
Raffinose	+	+	−	+	+	−
Inulin	+	+	−	−	−	+
Galactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+
Lactose	+	+	+	+	+	±
Dulcitol	−	−	−	−	−	−
Rhamnose	−	−	+	+	+	+
Trehalose	−	−	+	−	−	+
Sorbitol	−	−	−	−	−	−
Inositol	−	−	−	−	−	−
None	−	−	−	−	−	−

+, color of indicator changed to red. No coagulation.

−, no change in color of indicator.

Readings recorded were made at 9 days. No change occurred after this time, and the tubes were discarded at 14 days.

F. D. = Fort Defiance. L. = Leupp.

containing 0.5 cc. of a rich culture (5 days old on the semisolid medium) in water at temperatures varying from 52°C. to 65°C., and 5 and 10 minute exposures were made at each temperature. The whole amount of the heated culture was transferred, immediately after the heating, to fresh semisolid medium.

TABLE 10

Fermentation Tests. Dunham's Peptone Water Medium

	Aq. 1	D. 1	D. 2	D. 4A	D. 5	D. 6	D. 7	F.D. 4	L. 4
Dextrose	++++	++++	+++	++++	++++	++++	++++	+++	+++
Levulose	+++	++++	+++	+++	++++	+++	++++	+++	+++
Mannose	+++	++++	+++	+++	++++	+++	++++	+++	+++
Saccharose	+++	++++	+++	+++	+++	++++	++++	++	+++
Raffinose	−	−	−	−	−	−	−	±	
Inulin	+	±	+	±	+	±	+	−	−
Galactose	+++	++++	++	++	++++	+++	++++	+++	++
Maltose	+++	+++	+++	+++	++++	+++	++++	+++	++
Salicin	++++	++++	++++	++++	++++	++++	++++	++++	++++
Xylose	++	++++	+++	+++	++++	+++	++++	++++	+++
Mannitol	++++	++++	+++	+++	++++	++++	++++	+++	+++
Dextrin	++++	++++	+++	+++	++++	++++	++++	+++	+++
Arabinose	++++	++++	+	+	++++	++++	++++	++++	+++
Amygdalin	+++	++	+	+	+++	++++	+++	+++	+++
Lactose	+	+	+	+	+	±	±	+	+
Dulcitol	−	−	−	−	−	−	−	−	−
Rhamnose	+++	++++	+	+	+++	++++	++++	++++	+++
Trehalose	++	+++	+	−	+++	+++	+++	+++	++
Sorbitol	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−

Aq. = Albuquerque.
D. = Strains isolated by Thygeson in Denver.
F. D. = Fort Defiance.
L. = Leupp.

(*e*) *Effect of Cocaine.*—The organism is also sensitive to the action of cocaine hydrochloride, being killed in 5 minutes by 4 and 5 per cent solutions, and in 15 minutes by weaker solutions (2 and 3 per cent). Similar concentrations of procaine hydrochloride (novocain) have no inhibitory effect on its growth. In obtaining material for cultivation, therefore, it is desirable to avoid long exposure to high concentrations of cocaine.

SUMMARY AND CONCLUSIONS

One of the strains of *Bacterium granulosis* isolated by Noguchi in 1926 has been maintained in culture and in monkeys and continues to be capable, after 3 years, of inducing a chronic granular conjunctivitis in monkeys. Cultures of this strain have been recovered from the monkey lesions as late as 3 years after inoculation and have been shown to reproduce the granular disease in monkeys.

Six additional strains of *Bacterium granulosis* have been isolated from cases of trachoma occurring in the Indian schools of Arizona. The cultures thus obtained are identical morphologically and culturally with those isolated by Noguchi and have induced the same chronic granular conjunctivitis in monkeys. Advanced untreated cases are more favorable for cultural study than treated cases.

Cultures of *Bacterium granulosis* kept on semisolid medium containing 10 per cent rabbit serum ("leptospira medium") remain viable for many months at room temperature, and sealed ampoules of such cultures have been found to retain their pathogenicity for the monkey conjunctiva for at least 69 days at room temperature and at least 284 days at 4° to 6°C.

Several additional cultural and biological characteristics of *Bacterium granulosis* have been described. Of outstanding importance is the fact that cocaine, in contradistinction to novocain, has a bactericidal effect on the organism. This fact, in view of the common use of cocaine for anesthesia, may explain the negative results of cultivation experiments reported by some workers.

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EXPLANATION OF PLATE 26

FIG. 1. *M. rhesus* L-6, 19 days after inoculation with pooled cultures from Leupp cases.

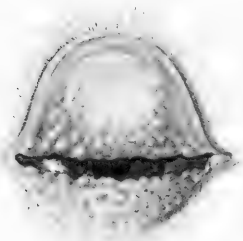
FIG. 2. *M. rhesus* 80, 2½ years after inoculation (1), showing presence of scar tissue.

FIG. 3. *M. rhesus* 5, 1½ years after inoculation, when the lid showed renewed activity of the lesions, and the left cornea became diffusely cloudy.

FIG. 4. *M. rhesus* 61, 57 days after inoculation with routine subcultures of Albuquerque Strain 1.

FIG. 5. *M. rhesus* 51, 65 days after inoculation with cultures recovered from *M. rhesus* 5.

Right Left



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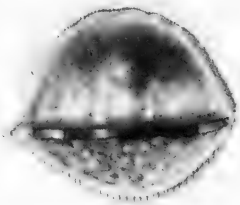

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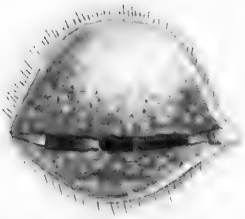

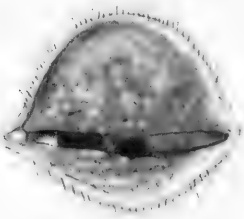

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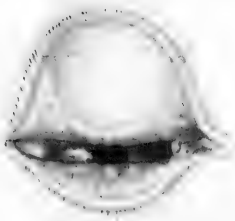

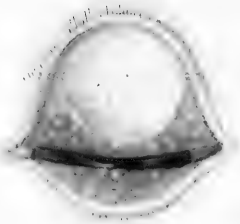

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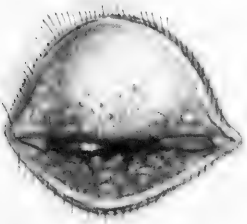

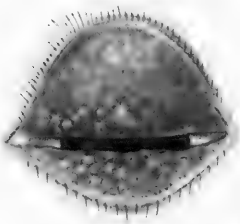

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(Tilden and Tyler: *Bacterium granulosis* and trachoma)

UNIV. OF MICH.

THE TRANSMISSION OF PERIODIC OPHTHALMIA OF HORSES BY MEANS OF A FILTERABLE AGENT

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Plates 27 to 31

(Received for publication, June 2, 1930)

In a previous communication (1) one of us together with Burky reported the results of a bacteriological study of periodic ophthalmia in horses. Both aerobic and anaerobic cultures of eyes freshly enucleated from horses with both active and quiescent periodic ophthalmia, were uniformly negative. The disease, however, has been shown by others to be definitely transmissible by inoculation into a normal eye of an exudate from an eye that was the seat of active disease. The purpose of this paper is to report the pathology of the disease as observed in the epidemic which we have investigated and to publish observations indicating that in this particular epidemic the disease was caused by a filterable agent.

In the fall of 1926 there occurred a sudden outbreak of periodic ophthalmia among thorough-bred horses on a farm located in high-rolling country in northern Maryland. The clinical appearance of the disease in this epidemic was identical with that observed elsewhere, and did not differ from that seen in sporadic cases which have come to our attention. The disease is essentially a recurrent serous uveitis. The onset is characterized by lachrymation, oedema of the periorbital fold, slight haziness of the cornea, the occurrence of a gelatinous, mucopurulent exudate in the anterior chamber, and the usual signs of iritis with synechiae and exudations into the vitreous. Exudates in the retina occurred, but were difficult to study on account of the disease of the anterior portions of the uvea.

The initial attack usually lasts from 4 to 10 days and then subsides, resulting at first in little permanent damage. The period of remission

is irregular, varying usually from 3 to 6 weeks, after which a fresh attack occurs. The attacks recur one after the other with increasing or decreasing severity. In a few horses the eyes recover from the attacks and show only small vitreous opacities, occasional posterior synechiae and varying amounts of retinal atrophy. In a few of the horses in which such recovery occurred there was almost complete retinal atrophy with resultant blindness, while in others the only evidence of the disease was limited to several vitreous opacities. The commonest outcome, however, is for the attacks to occur with increasing severity, with the formation of complete posterior annular synechiae, secondary glaucoma, cataract and ultimately moderate phthisis bulbi.

Pathology

The pathology of the disease has been described by several authors, and most recently by Heinrich Jakob (2). These descriptions, however, do not correspond exactly with the picture we have constantly observed in the horses studied.

The characteristic lesion we have observed appears to be a focal mononuclear infiltration of the uveal tract, the pigment epithelium, the retina and the optic nerve.

This focal infiltration spreads, cyclitic membranes may form, and scarring with complete detachment of the retina may take place. The mononuclear infiltration occurs throughout the optic nerve and in focal points throughout the retina. In advanced, severe cases a heavy cyclitic membrane covers the ciliary process. The cyclitic membrane is densely infiltrated with small round cells. Follicular accumulations of round cells, which superficially resemble lymph follicles, are found throughout the stroma of the uveal tract and in the cyclitic membrane. The essential features of the pathological picture are illustrated in the micro-photographs.

Figs. 1, 2, 3, 6, 7, 10, 11 show, under different magnifications, typical lesions from a number of different horses whose vision was destroyed by the natural disease.

One of the most notable features in the pathological picture is the formation of follicular collections of round cells, which superficially resemble lymph-follicles. These may occur in the stroma of the iris, or outside the normal tissues of the eye, lying in the inflammatory cyclitic membrane. Fig. 12 shows the typical appearance of these follicular collections of cells. While these collections superficially resemble lymph-follicles, they appear, on closer inspection, to differ from them. They have no germinal centers; capillaries are present, and both large and small mononuclear

cells are found, together with plasma and epithelioid cells. Further, we could find no sinuses, such as are found in true lymph-follicles. Stained with the Foot stain, the reticulum characteristic of true lymph-follicles could not be demonstrated.

Whether the follicular collections of cells are pathognomonic of this disease cannot be said as yet. They have been constantly observed in all the horses with periodic ophthalmia that we have studied, and we have not found them in the eyes of horses with uveitis caused by bacteria. Further study of uveitis in horses produced by other agents will be necessary to determine whether or not the collections of cells are to be considered as a specific feature of periodic ophthalmia.

EXPERIMENTAL

Our first studies on the etiology of this disease were concerned with an attempt to cultivate a bacterium from the diseased eyes. All aerobic and anaerobic cultures from eyes showing the disease in both active and quiescent form were entirely negative. We were forced therefore to disagree with Rosenow's (3) conclusion that a Gram negative flavobacterium is the etiological agent of the disease. After numerous attempts to demonstrate other possible causes such as spirochetes, plasmodia, and tuberculin hypersensitivity, none of which were successful, we considered the possibility of a filterable agent as the specific etiologic factor.

A horse in the acute stage of the disease was sacrificed and the eyes removed. One eye was saved for histological study, and the other, affected with the disease in the acute stage, was opened under aseptic precautions, the aqueous and vitreous humors as well as the retina and uveal tract removed and ground up in a sterile mortar with sterile sand and several cubic centimeters of physiological salt solution. The supernatant fluid was then passed through a Berkefeld N filter. This filtrate was tested for sterility by aerobic and anaerobic cultures on rabbit blood agar, glucose beef infusion broth and cooked meat medium under vaseline seal. Under butyn anaesthesia 0.5 cc. of this filtrate was then injected into the vitreous humor of a normal horse. Within 24 hours the horse developed the clinical picture typical of periodic ophthalmia, with oedema of the periorbital fold, lachrymation, photophobia, ciliary congestion, exudation in the anterior chamber, vitreous opacities and the evidence of an acute iritis. The attack lasted about 10 days, then subsided and recurred several times.

This experiment indicated that it was possible to recover from the eyes of horses with periodic ophthalmia a filter-passing agent which

was capable of producing the clinical picture of the disease in a normal horse, but gave no indication as to whether or not the agent was a living virus. In order to throw light on this point it was decided to attempt to transmit the disease to other species with the hope of carrying it through several transfer-generations of animals, because of the expense entailed in the use of horses. Rabbits were selected for this purpose, and a second horse with periodic ophthalmia in the acute stage was sacrificed and the eyes enucleated. One eye was saved for histological study and a filtrate was prepared from the second eye in the manner described above. Inasmuch as the same technique for preparing and testing the filtrate for sterility was used throughout all the transmission experiments we shall, for the sake of brevity, refer to these procedures as the "usual technique." The clear filtrate, proven sterile by culture, was injected directly into the vitreous of each eye of a series of six rabbits. All injections were made while the rabbits were under the influence of an anaesthetic.

There quickly developed in these rabbits a clinical picture which, while not similar in every respect to that observed in horses, was nevertheless quite constant.

There was no visible involvement of the anterior uvea, but within 24 hours there developed an acute retinitis with fluffy exudates throughout the retina. In some of the animals the lesions tended to recur after a remission of 2 to 3 weeks. The histology of these lesions is described below. Control inoculations into normal rabbits' eyes with filtrate of emulsions from normal horses' eyes failed to produce such lesions, and aerobic and anaerobic cultures of the diseased eyes were sterile.

These facts warranted the surmise that the disease had been transmitted successfully to rabbits by the intra-vitreous injection of a filtrate from the eyes of a horse with periodic ophthalmia.

Acting on this assumption, an attempt was next made to carry the disease from the rabbit back to the horse.

A normal horse (No. 2) was inoculated directly into the vitreous with 1 cc. of a filtrate prepared according to the usual technique from one diseased eye of each of three of the experimentally infected rabbits. Within 24 hours after inoculation the horse showed the typical picture of periodic ophthalmia observed in horses with the natural disease. The acute symptoms persisted for 10 days and then subsided. 3 weeks later the disease recurred and there was subsequently a second recurrence.

This experiment suggested that the filterable agent was capable of propagation in the rabbit's eye, but as the transmission had been made through a single transfer-generation of rabbits from the naturally diseased horse to the experimentally infected eye of the horse, it was necessary to elaborate the original experiment in order to establish this theory.

An attempt was made therefore to transmit the disease from horses to rabbits and to carry it through several transfer-generations of rabbits in succession before making the crucial experiment of transmitting it to the horse.

Accordingly another horse with periodic ophthalmia was sacrificed and a filtrate prepared with the usual technique from the acutely diseased eye. This filtrate, proven sterile by aerobic and anaerobic cultures, was injected into the vitreous of each eye of a series of four rabbits. All four rabbits developed a retinitis similar to that observed in the preceding experiments. After an interval of 2 weeks, two of these rabbits were sacrificed, the eyes removed, cultured by both aerobic and anaerobic methods, and a filtrate prepared from them. A second series of four rabbits was inoculated by intravitreous injection with this filtrate. The remaining two rabbits were kept for further observation and for histological study.

In this manner the disease was carried through six successive transfer-generations of rabbits with the constant occurrence of the typical retinitis. Aerobic and anaerobic cultures were made of all eyes removed for preparation of filtrates. These cultures were uniformly negative. Occasionally a rabbit was encountered which showed no lesions after inoculation. Such rabbits were not used for transfer. These rabbits which appeared refractory to inoculation were the exception, for the occurrence of the retinitis was remarkably constant throughout.

A filtrate from the eyes of three rabbits of the sixth transfer-generation was then inoculated into the vitreous of the left eye of each of two normal horses (Nos. 3 and 4). Both of these horses developed a clinical picture similar to that observed in the natural disease.

The initial symptoms were oedema of the periorbital fold, lachrymation, photophobia, ciliary congestion and the occurrence of a gelatinous, mucopurulent exudate in the anterior chamber. The signs of inflammation were noted 24 hours after inoculation, and they reached their height within 48 hours. The initial attack lasted from 1 to 2 weeks and then diminished, leaving only slight evidence

of inflammation. In each horse the disease recurred after periods of from 2 to 4 weeks. One horse (No. 3) was apparently very susceptible. The injected eye showed an unusually severe reaction. The attacks recurred with increasing severity, and the final picture was similar to that observed in cases of severe natural disease—complete posterior synechiae, cataractous lens, and moderate phthisis. The other horse (No. 4) had four attacks, each less severe than the preceding one. The attacks finally ceased spontaneously, and the picture was then similar to that observed in the eyes of horses in which the natural disease had healed normally, as the only evidence of the disease was some clouding of the vitreous and a few posterior synechiae. None of the experimental horses had any symptoms of involvement of the control or uninjected eye.

Controls

The following control experiments were performed.

1. A filtrate was prepared in the usual manner from the eye of a normal horse and injected into the vitreous of each eye of four rabbits. The eyes of these animals did not exhibit the retinitis observed in the eyes of rabbits injected with filtrates from the eyes of horses with periodic ophthalmia. Histological study of these eyes showed no pathological lesions.

2. The eye of a normal horse was injected with 2 cc. of sterile filtrate obtained from normal rabbits' eyes. This horse showed a moderate reaction following the injection which lasted about 48 hours. Within 4 days the eye appeared entirely normal, and remained so for a period of 3 months observation. The horse was then sacrificed. Histological examination of the inoculated eye showed no pathological lesions resembling, even remotely, those of periodic ophthalmia.

3. In order to exclude a possibility that the experimental lesions were due to traumatism alone, salt solution was injected into the vitreous of the eyes of normal rabbits and of a normal horse. Clinical and histological examination of these eyes revealed no pathological lesions.

Pathology in Experimental Rabbits

The pathological lesions found in the experimental rabbits were less extensive than those found in the horses. They consisted chiefly in focal areas of acute retinal inflammation with disorganization of the normal retinal picture by round cell infiltration, which was fundamentally the same as the pathological lesion found in horses. No follic-

ular collections of cells were observed. These lesions were seen both in the acute stage and the stage of repair. Figs. 8 and 9 show such lesions. Occasionally lesions in the choroid were found, although as a rule the uveal tract was remarkably free from abnormalities.

The optic nerve was also involved in a few rabbits showing mononuclear infiltration of the stroma and of the tissues over the surface of the nerve. Fig. 13 illustrates such lesions.

One rabbit showed a definite infiltration in the sheath of the optic nerve, such as is found in meningitis, the cells near the nervehead being chiefly mononuclear in type, while polynuclear cells were found posteriorly in the sheath of the nerve (Fig. 4).

We have as yet made no intra-cerebral injections of this filterable agent, and the brains of the experimental rabbits and horses have not yet been studied, so that we have no information as to the condition of the central nervous system.

Pathology of Disease in the Inoculated Horse

The lesions in the disease produced experimentally were uniformly of the same character as those found in the natural disease.

Figs. 5 and 14 to 17 illustrate the various pathological lesions found in Horse 3 in which the attack recurred with increasing severity until the disease reached the end stage: mononuclear infiltration of the iris with collections of round cells (Fig. 14); acute lesions of the choroid and retina with proliferative changes, and disorganization and infiltration of the retina by mononuclear cells (Fig. 15); follicular-like collections, chiefly of mononuclear cells, lying both in the stroma of the anterior uvea and in the cyclitic membrane (Figs. 16 and 17) and optic neuritis with mononuclear infiltration of the nerve stroma (Fig. 5).

The eyes of the other two horses (Nos. 2 and 4) in which the clinical picture of the disease was produced experimentally, finally healed, and at the time the horses were sacrificed the eyes showed but little clinical abnormality. Pathological study of the eyes of these horses, as might be expected, did not show the pronounced changes found in the eyes which were the seat of advanced disease.

One horse (No. 2) showed several focal areas of round cell infiltration throughout the stroma of the anterior uvea, while the other horse (No. 4) showed some diffuse cellular infiltration around the pectinate ligament and the root of the ciliary processes. We have, unfortunately, no histological preparations of eyes from horses that have recovered from the natural disease, but clinical examination of such eyes gives no reason to suppose that more extensive pathological lesions could be found than these illustrations show. The lesions found in these two

horses illustrated in Figs. 18 and 19, were what might be expected in horses in which the disease had healed after several acute attacks.

COMMENT

These experiments were designed to solve the problem of whether periodic ophthalmia in horses can be successfully transmitted by means of a filter-passing agent. We believe it has been. The reasons upon which we base our belief are three-fold. First, the experimental disease is indistinguishable clinically from the disease as it is observed in nature, not only in the actual appearance of the lesions but in the relapsing course which they pursue. Secondly, the pathological lesions in the experimental disease and in the natural disease are identical. Thirdly, we could find no evidence that bacteria produced these lesions. Aerobic and anaerobic cultures made from eyes in the acute stage of the disease gave uniformly negative results, and sections of such eyes, stained by special methods did not show either bacteria or spirochetes. Furthermore, the infiltration with mononuclear cells, and the almost complete absence of polymorphonuclear cells in the exudates would be unusual in a disease caused by bacteria such as are usually found in pyogenic inflammations. We believe therefore these experiments to indicate that this epidemic of periodic ophthalmia in horses is caused by a filterable agent, and that the disease can be transmitted to other animals by the intra-vitreous inoculation of this agent. The successful transmisson of the disease to the horse after successive transfers through six series of rabbits indicates that the agent is capable of propagation in this species and belongs to the group of filterable viruses. In the main this agent appears to attack the optic nerve, retina, pigment epithelium, and the uveal tract. Whether the involvement of the uveal tract is an extension of the inflammation from the retina and pigment epithelium of the ciliary processes cannot be stated. In rabbits the disease produced by this filterable agent is limited almost entirely to the nervous structures of the eye, while in horses the onset and involvement of the anterior uvea are so sudden and abrupt that it is impossible to tell where the initial lesion starts.

It is also by no means certain that the action of this filterable agent is limited solely to the eyes. Two horses and a number of rabbits

were autopsied and sections of the viscera studied histologically, but no lesions were found. The brains and spinal cords, however, were not examined in any of the animals. Sections of the optic nerves, both of the horses and rabbits, suggest the possibility that disease of the central nervous system may also occur. None of the horses under observation died from natural causes, but there was a low mortality among the experimental rabbits. It has been noted that horses affected with the disease appear irritable and tend to throw their heads up unduly. The rabbits were observed at regular intervals, but we did not see in them any symptoms of central nervous system involvement. We can therefore hazard no opinion as to this possibility, other than that the sections of the eyes suggest that there may have been such involvement. This is a point for future investigation.

We have not been able to demonstrate inclusion bodies. In a number of sections we have found granules in cells which suggested inclusion bodies, but in no instance could these be definitely identified as such. An explanation of our failure to find such bodies may be that they are found chiefly in the early stages of diseases caused by filterable agents; our sections of eyes with the natural disease were all obtained from blind horses in which the disease had reached the end stage. Likewise, our experimental horses were all in the late or healed stages of the disease at the time of autopsy, for in the transmission experiments it was necessary to observe the horses for a long period of time in order to establish the clinical picture of recurrent attacks.

Not all horses appear uniformly susceptible to the disease. The morbidity rate in a herd is relatively low. There was a marked variation in the severity of the clinical picture both in the natural disease and in that experimentally induced. While the eyes of a few horses affected with the natural disease eventually healed with little or no final damage to the eyes, the disease usually progressed until there was more or less complete scarring of the eyes with concomitant loss of vision. The comparative mildness of the experimental disease in a few instances may possibly be attributable to a partial natural immunity. The low morbidity of the disease in a herd is in harmony with this conception. Moreover, not all rabbits appear to be susceptible to the experimental disease. A few were encountered which showed no reaction to the intra-vitreous injection of the filterable agent.

We have no knowledge of the mode of infection or the natural method of transmission of the disease. Intravenous inoculation of filtrates into four rabbits gave negative results. We cannot state whether or not this filterable agent is similar to any of the other filterable viruses so far studied, or if it constitutes a specific entity in itself. We have as yet made no studies on the possible protective or therapeutic properties of convalescent serum. These are all points for further investigation.

CONCLUSIONS

A filterable agent has been obtained from the humors and tissues of the eyes of horses suffering from active periodic ophthalmia. The intra-vitreous injection of this filtrate produced in normal horses the same clinical and pathological picture observed in the natural disease. This filtrate injected into rabbits produced a different clinical picture, but the essential pathological lesions closely resembled those found in horses. After passage of the filterable agent through six generations of rabbits, it again produced the clinical and pathological picture of the natural disease when injected into the eyes of normal horses.

It appears, in this epidemic at least, that this filterable agent was the specific etiological factor of the periodic ophthalmia.

In conclusion we desire to express our thanks to Major G. L. Stryker for the gift of horses with the disease, and for his cooperation and assistance throughout the course of this investigation, without which it could not have been conducted. We are indebted to Dr. F. B. Kindell and Dr. S. S. Blackman for autopsy examination of horses, to Dr. Jonas Friedenwald and Dr. F. H. Verhoeff for their valuable assistance in the examination of the histological specimens of the eyes, and to Dr. T. B. Turner for aid in the manipulations incident to transfer.

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EXPLANATION OF PLATES

PLATE 27

FIG. 1. (Natural disease.) Mononuclear infiltration of stroma of iris and follicular accumulation of round cells (high power)

FIG. 2. (Natural disease.) Monocellular infiltration over ciliary processes.

FIG. 3. (Natural disease.) Optic neuritis.

FIG. 4. (Experimental disease in rabbit.) Mononuclear infiltration in sheath of optic nerve.

FIG. 5. (Experimental disease in Horse 3.) Optic neuritis with mononuclear infiltration of nerve stroma.

PLATE 28

FIG. 6. (Natural disease.) Focal lesion in retina; mononuclear infiltration.

FIG. 7. (Natural disease.) Disorganization and atrophy of retina in advanced disease.

FIG. 8. (Experimental disease in rabbit.) Acute lesion of retina.

FIG. 9. (Experimental disease in rabbit.) Healing lesion of retina with fibrosis.

PLATE 29

FIG. 10. (Natural disease.) Acute lesion over pigment epithelium and follicular accumulation of mononuclear cells.

FIG. 11. (Natural disease.) Mononuclear infiltration of choroid.

FIG. 12. (Natural disease.) Follicular accumulation of mononuclear cells (low power).

FIG. 13. (Experimental disease in rabbit.) Optic neuritis.

PLATE 30

FIG. 14. (Experimental disease in Horse 3.) Mononuclear infiltration of iris and follicular collection of cells.

FIG. 15. (Experimental disease in Horse 3.) Acute lesion in choroid with dense mononuclear infiltration.

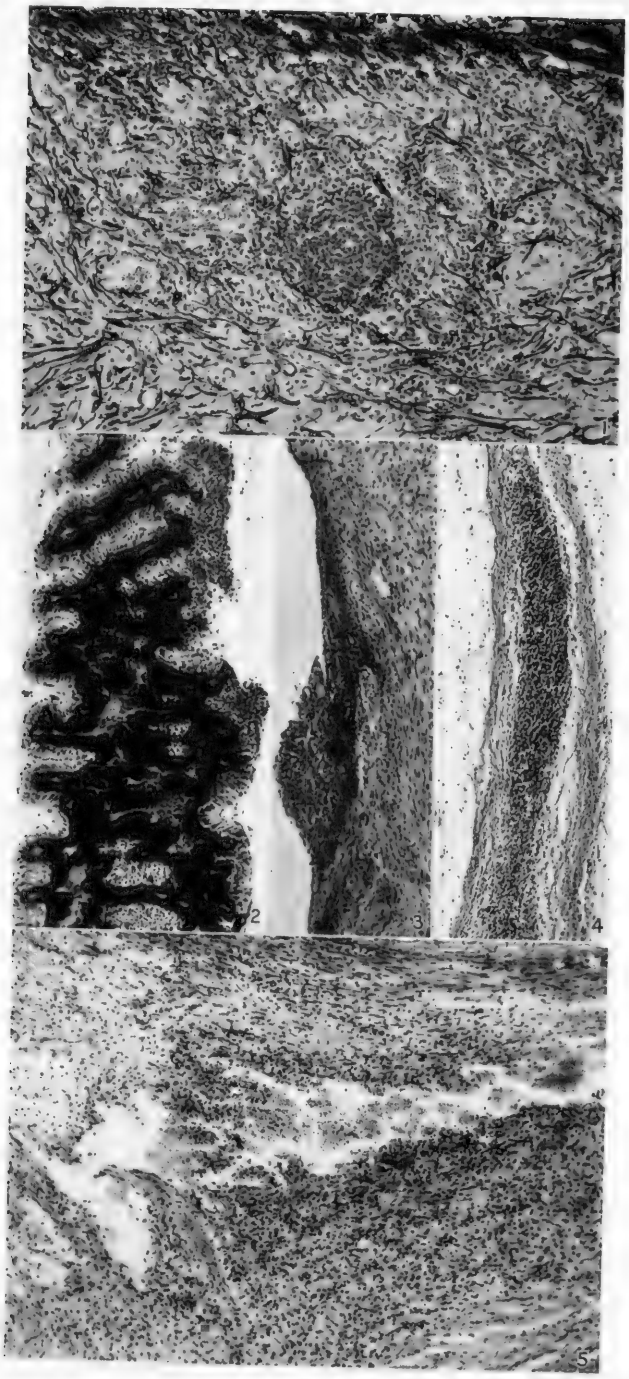
FIG. 16. (Experimental disease in Horse 3.) Infiltration and follicular accumulation of mononuclear cells in anterior uvea (low power).

PLATE 31

FIG. 17. (Experimental disease in Horse 3.) Follicular-like collection of mononuclear cells lying in the inflammatory cyclitic membrane (low power).

FIG. 18. (Experimental disease in Horse 2.) Focal area of mononuclear infiltration in anterior uvea (high power).

FIG. 19. (Experimental disease in Horse 4.) Diffuse mononuclear infiltration about pectinate ligament.



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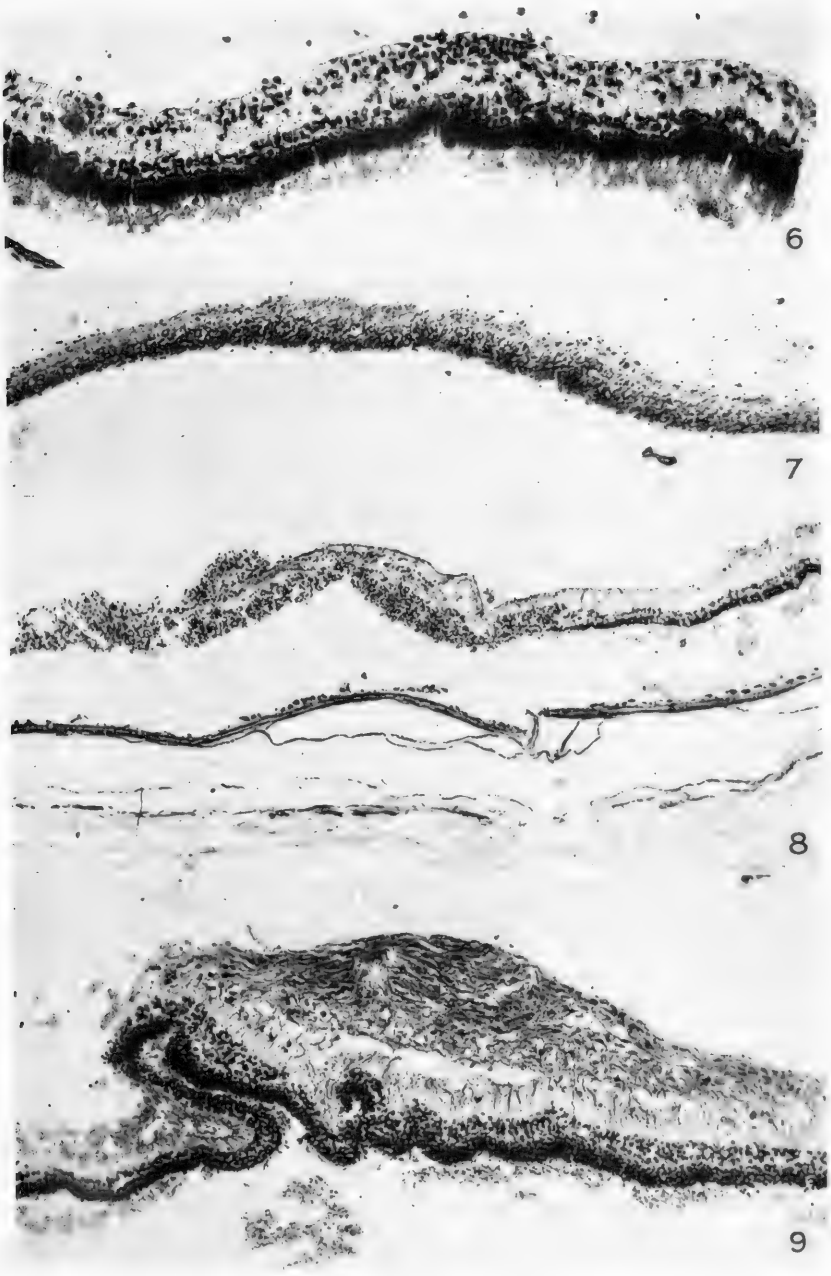

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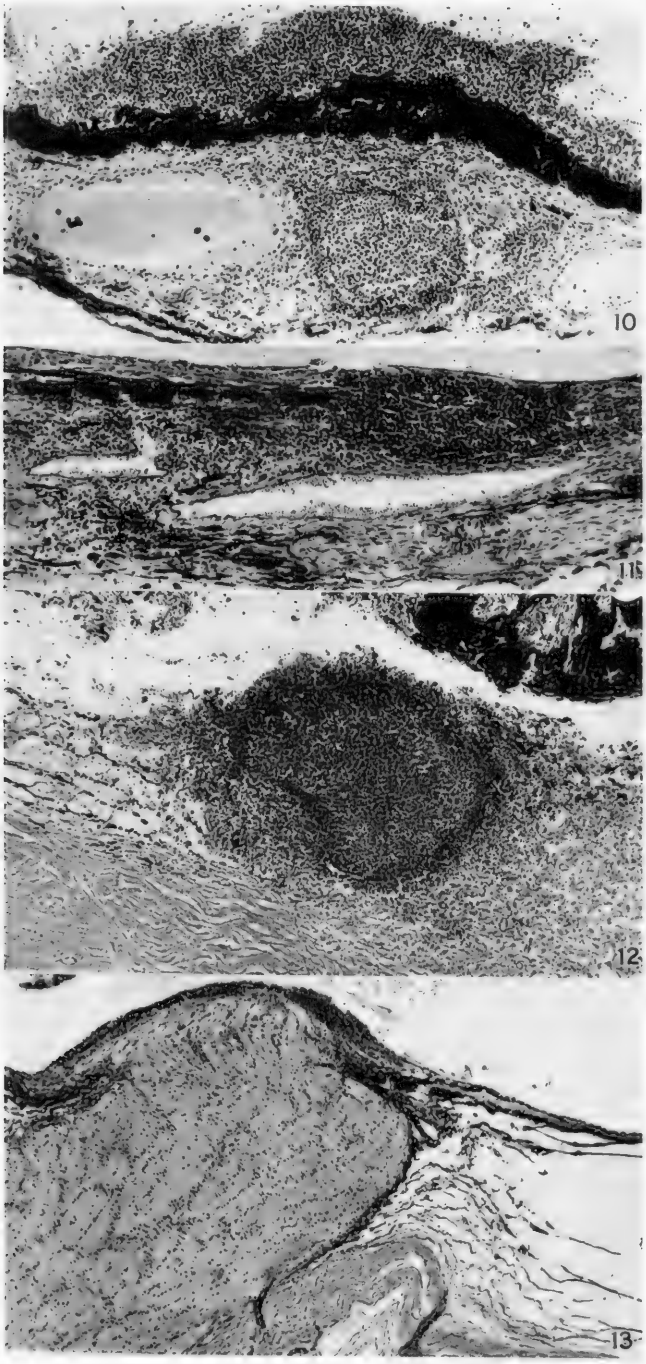

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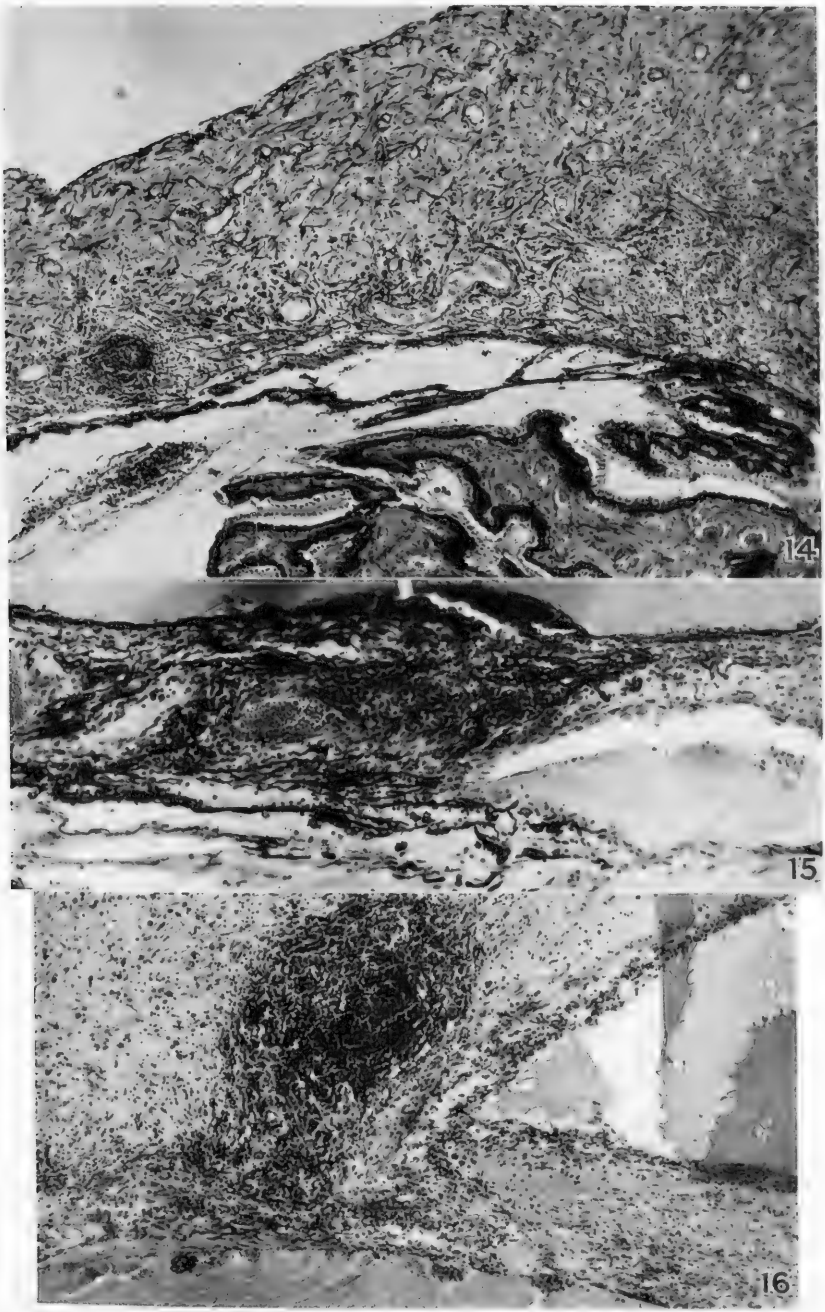

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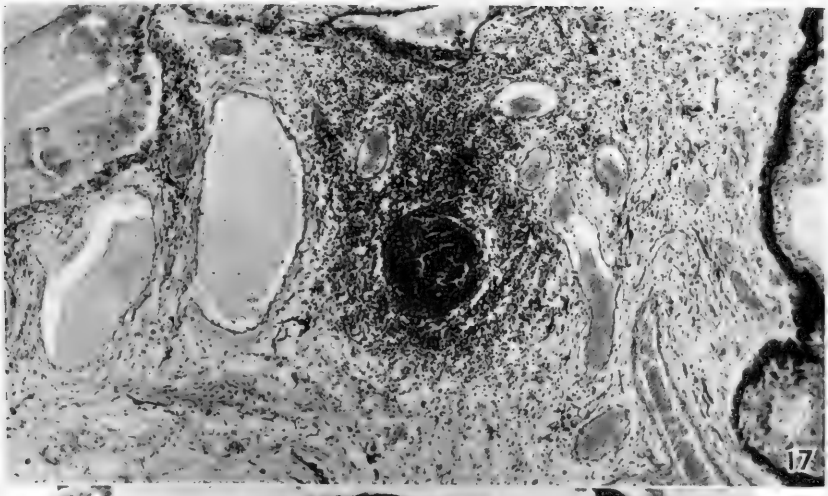

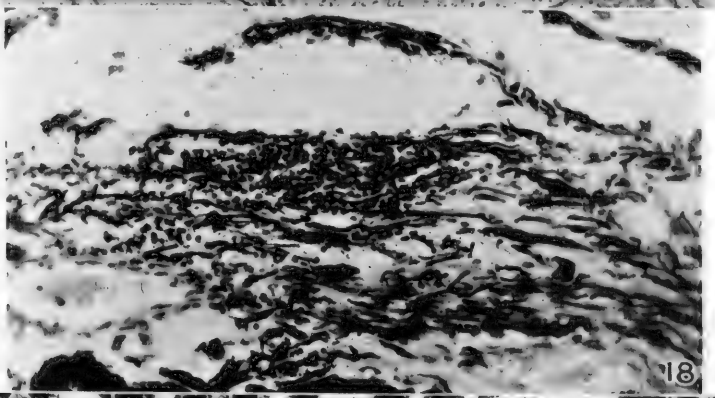

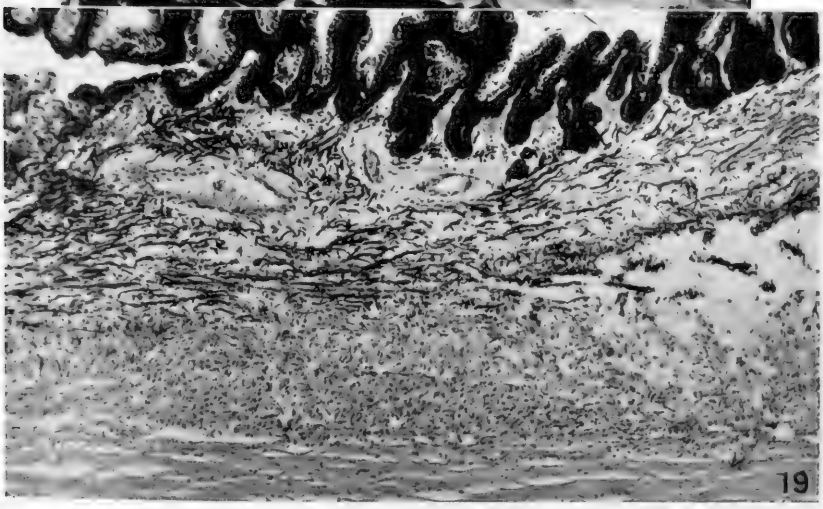

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